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EPA 0235410 EPA 0234075
Biochemistry Society Transactions 1987 Vol 15 No pages 17-18
Journal of Molecular Biology 1987 Vol 193 No 1 pages 41-46
Proceedings of the National Academy of Sciences (USA) 1987 Vol 83 No 22 pages 8447-8451
Gene 1985 Vol 34 No 1 pages 105-110
Journal of Bacteriology 1982 Vol 149 No 2 pages 587-594

(58) Field of search
C3H
Selected US specifications from IPC sub-class C12N

(54) Gene fusion comprising β-glucuronidase

(57) A gene fusion product comprises a gene coding for beta-glucuronidase (GUS), eg the *uidA* gene of *E. coli*, and can be used in the introduction, monitoring and regulation of expression of a desired gene in hosts such as plants, animals, yeasts etc. Plasmids containing the beta-glucuronidase gene and N-terminal sites for insertion and fusion of desired gene are disclosed.

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The drawings 6 and 7 originally filed were informal and the print here reproduced is taken from a later filed formal copy.

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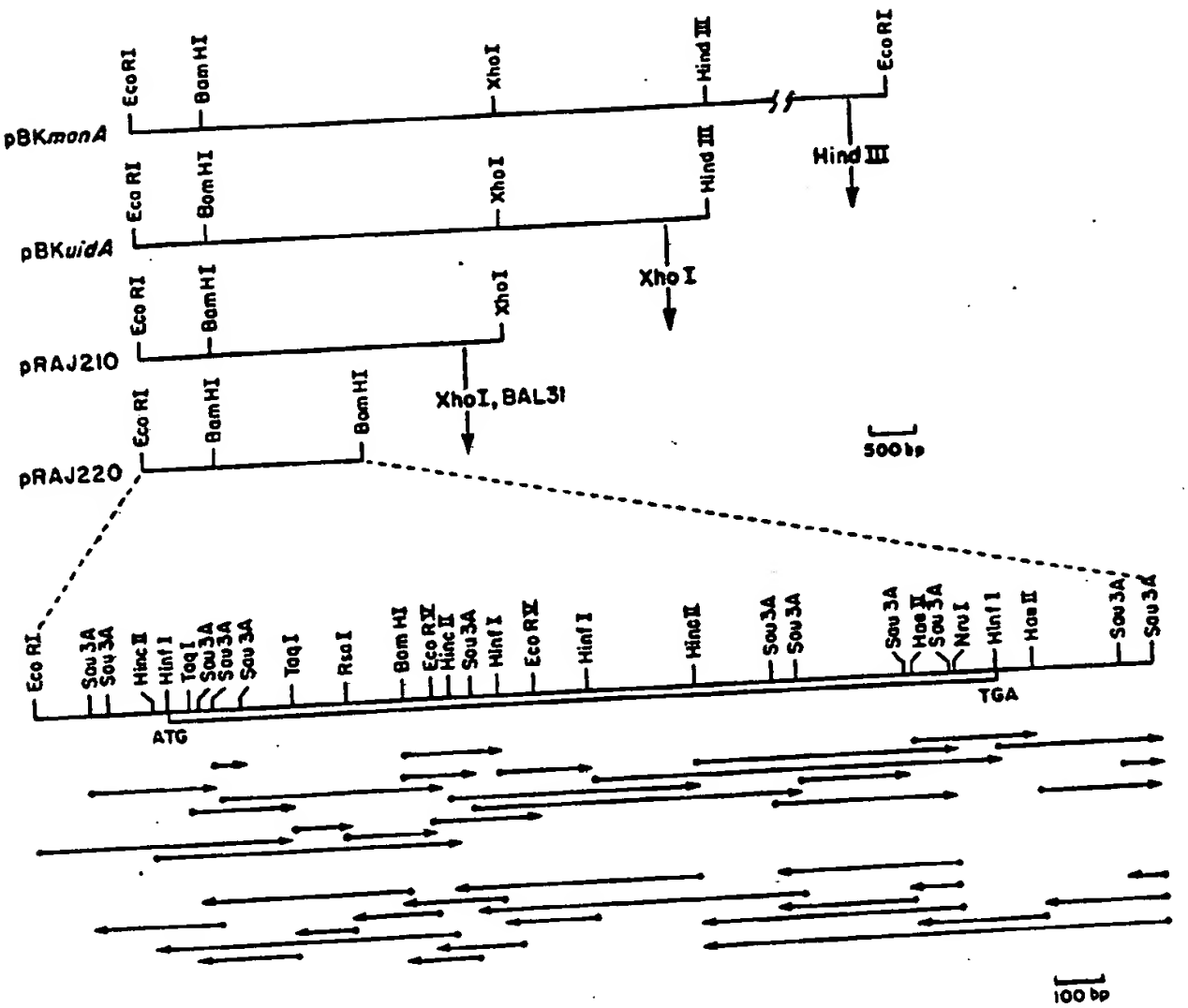

FIG 1

FIG 2A

GAATTCCCCCCAAAATATTCACTGTAGCCATATGTCATGAGAGTTTATCGTTCCCAATACGCTCGAACGAACGTTCGGTTGCTTATTTTATGGCTTCTGTCAACGCTGTTTTAAAGATTA
1 50 100

ATGCGATCTATATCAGGCTGTGGGTATTGCAGTTTTTGGTTTTTTGATCGCGGTGTCAGTTCTTTTTATTTCCATTTCTGTTCCATGGGTTTCTCACAGATAACTGTGTGCAACACAGA
150 200

ATTGGTTAACTAATCAGATTAAAGGTTGACCAGTATTATTATCTTAATGACGAGTCCCTT Met Leu Arg Pro Val Glu Thr Pro Thr Arg Glu Ile Lys Lys Leu
250 ATG TTA CGT CCT GTA GAA ACC CCA ACC CGT GAA ATC AAA AAA CTC
300

Asp Gly Leu Trp Ala Phe Ser Leu Asp Arg Glu Asn Cys Gly Ile Asp Gln Arg Trp Trp Glu Ser Ala Leu Gln Glu Ser Arg Ala Ile
GAC GGC CTG TGG GCA TTC AGT CTG GAT CGC GAA AAC TGT GGA ATT GAT CAG CGT TGG TGG GAA AGC GCG TTA CAA GAA AGC CGG GCA ATT
350 400

Ala Val Pro Gly Ser Phe Asn Asp Gln Phe Ala Asp Ala Asp Ile Arg Asn Tyr Ala Gly Asn Val Trp Tyr Gln Arg Glu Val Phe Ile
GCT GTG CCA GGC AGT TTT AAC GAT CAG TTC GCC GAT GCA GAT ATT CGT AAT TAT GCG GGC AAC GTC TGG TAT CAG CGC GAA GTC TTT ATA
450 500

Pro Lys Gly Trp Ala Gly Gln Arg Ile Val Leu Arg Phe Asp Ala Val Thr His Tyr Gly Lys Val Trp Val Asn Asn Gln Glu Val Met
CCG AAA GGT TGG GCA GGC CAG CGT ATC GTG CTG CGT TTC GAT GCG GTC ACT CAT TAC GGC AAA GTG TGG GTC AAT AAT CAG GAA GTG ATG
550 600

Glu His Gln Gly Gly Tyr Thr Pro Phe Glu Ala Asp Val Thr Pro Tyr Val Ile Ala Gly Lys Ser Val Arg Ile Thr Val Cys Val Asn
GAG CAT CAG GGC GGC TAT ACG CCA TTT GAA GCC GAT GTC ACG CCG TAT GTT ATT GCC GGG AAA AGT GTA CGT ATC ACC GTT TGT GTG AAC
650 700

Asn Glu Leu Asn Trp Gln Thr Ile Pro Pro Gly Met Val Ile Thr Asp Glu Asn Gly Lys Lys Lys Gln Ser Tyr Phe His Asp Phe Phe
AAC GAA CTG AAC TGG CAG ACT ATC CCG CCG GGA ATG GTG ATT ACC GAC GAA AAC GGC AAG AAA AAG CAG TCT TAC TTC CAT GAT TTC TTT
750

Asn Tyr Ala Gly Ile His Arg Ser Val Met Leu Tyr Thr Thr Pro Asn Thr Trp Val Asp Asp Ile Thr Val Val Thr His Val Ala Gln
AAC TAT GCC GGG ATC CAT CGC AGC GTA ATG CTC TAC ACC ACG CCG AAC ACC TGG GTG GAC GAT ATC ACC GTG GTG ACG CAT GTC GCG CAA
800 850

Asp Cys Asn His Ala Ser Val Asp Trp Gln Val Val Ala Asn Gly Asp Val Ser Val Glu Leu Arg Asp Ala Asp Gln Gln Val Val Ala
GAC TGT AAC CAC GCG TCT GTT GAC TGG CAG GTC GTG GCC AAT GGT GAT GTC AGC GTT GAA CTG CGT GAT GCG GAT CAA CAG GTG GTT GCA
900 950

Thr Gly Gln Gly Thr Ser Gly Thr Leu Gln Val Val Asn Pro His Leu Trp Gln Pro Gly Glu Gly Tyr Leu Tyr Glu Leu Cys Val Thr
ACT GGA CAA GGC ACT AGC GGG ACT TTG CAA GTG GTG AAT CCG CAC CTC TGG CAA CCG GGT GAA GGT TAT CTC TAT GAA CTG TGC GTC ACA
1000 1050

Ala Lys Ser Gln Thr Glu Cys Asp Ile Tyr Pro Leu Arg Val Gly Ile Arg Ser Val Ala Val Lys Gly Glu Gln Phe Leu Ile Asn His
GCC AAA AGC CAG ACA GAG TGT GAT ATC TAC CCG CTT CGC GTC GGC ATC CGG TCA GTG GCA GTG AAG GGC GAA CAG TTC CTG ATT AAC CAC
1100 1150

Lys Pro Phe Tyr Phe Thr Gly Phe Gly Arg His Glu Asp Ala Asp Leu Arg Gly Lys Gly Phe Asp Asn Val Leu Met Val His Asp His
AAA CCG TTC TAC TTT ACT GGC TTT GGT CGT CAT GAA GAT GCG GAC TTA CGT GGC AAA GGA TTC GAT AAC GTG CTG ATG GTG CAC GAC CAC
1200

Ala Leu Met Asp Trp Ile Gly Ala Asn Ser Tyr Arg Thr Ser His Tyr Pro Tyr Ala Glu Glu Met Leu Asp Trp Ala Asp Glu His Gly
GCA TTA ATG GAC TGG ATT GGG GCC AAC TCC TAC CGT ACC TCG CAT TAC CCT TAC GCT GAA GAG ATG CTC GAC TGG GCA GAT GAA CAT GGC
1250 1300

Ile Val Val Ile Asp Glu Thr Ala Ala Val Gly Phe Asn Leu Ser Leu Gly Ile Gly Phe Glu Ala Gly Asn Lys Pro Lys Glu Leu Tyr
ATC GTG GTG ATT GAT GAA ACT GCT GCT GTC GGC TTT AAC CTC TCT TTA GGC ATT GGT TTC GAA GCG GGC AAC AAG CCG AAA GAA CTG TAC
1350 1400

Ser Glu Glu Ala Val Asn Gly Glu Thr Gln Gln Ala His Leu Gln Ala Ile Lys Glu Leu Ile Ala Arg Asp Lys Asn His Pro Ser Val
AGC GAA GAG GCA GTC AAC GGG GAA ACT CAG CAA GCG CAC TTA CAG GCG ATT AAA GAG CTG ATA GCG CGT GAC AAA AAC CAC CCA AGC GTG
1450 1500

Val Met Trp Ser Ile Ala Asn Glu Pro Asp Thr Arg Pro Gln Val His Gly Asn Ile Ser Pro Leu Ala Glu Ala Thr Arg Lys Leu Asp
GTG ATG TGG AGT ATT GCC AAC GAA CCG GAT ACC CGT CCG CAA GTG CAC GGG AAT ATT TCG CCA CTG GCG GAA GCA ACG CGT AAA CTC GAC
1550 1600

FIG 2

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Pro Thr Arg Pro Ile Thr Cys Val Asn Val Met Phe Cys Asp Ala His Thr Asp Thr Ile Ser Asp Leu Phe Asp Val Leu Cys Leu Asn
CCG ACG CGT CCG ATC ACC TGC GTC AAT GTA ATG TTC TGC GAC GCT CAC ACC GAT ACC ATC AGC GAT CTC TTT GAT GTG CTG TGC CTG AAC
                                                            1650

Arg Tyr Tyr Gly Trp Tyr Val Gln Ser Gly Asp Leu Glu Thr Ala Glu Lys Val Leu Glu Lys Glu Leu Leu Ala Trp Gln Glu Lys Leu
CGT TAT TAC GGA TGG TAT GTC CAA AGC GGC GAT TTG GAA ACG GCA GAG AAG GTA CTG GAA AAA GAA CTT CTG GCC TGG CAG GAG AAA CTG
    1700                                                    1750

His Gln Pro Ile Ile Ile Thr Glu Tyr Gly Val Asp Thr Leu Ala Gly Leu His Ser Met Tyr Thr Asp Met Trp Ser Glu Glu Tyr Gln
CAT CAG CCG ATT ATC ATC ACC GAA TAC GGC GTG GAT ACG TTA GCC GGG CTG CAC TCA ATG TAC ACC GAC ATG TGG AGT GAA GAG TAT CAG
                    1800                                                    1850

Cys Ala Trp Leu Asp Met Tyr His Arg Val Phe Asp Arg Val Ser Ala Val Val Gly Glu Gln Val Trp Asn Phe Ala Asp Phe Ala Thr
TGT GCA TGG CTG GAT ATG TAT CAC CGC GTC TTT GAT CGC GTC AGC GCC GTC GTC GGT GAA CAG GTA TGG AAT TTC GCC GAT TTT GCG ACC
                                1900                                                    1950

Ser Gln Gly Ile Leu Arg Val Gly Gly Asn Lys Lys Gly Ile Phe Thr Arg Asp Arg Lys Pro Lys Ser Ala Ala Phe Leu Leu Gln Lys
TCG CAA GGC ATA TTG CGC GTT GGC GGT AAC AAG AAA GGG ATC TTC ACT CGC GAC CGC AAA CCG AAG TCG GCG GCT TTT CTG CTG CAA AAA
                                        2000                                                                    2050

Arg Trp Thr Gly Met Asn Phe Gly Glu Lys Pro Gln Gln Gly Gly Lys Gln OP
CGC TGG ACT GGC ATG AAC TTC GGT GAA AAA CCG CAG CAG GGA GGC AAA CAA TGA ATCAACAACTCTCCTGGCGCACCATCGTCGGCTACAGCCTCGGTGACGT
                                                        2100            ORF →                                           2150

CGCCAATAACTTCGCCTTCGCAATGGGGGCGCTCTTCCTGTTGAGTTACTACACCGACGTCGCTGGCGTCGGTGCCGCTGCCGCGGCACATGCTGTTACTGGTGCGGGTATTCGATGCCTT
                                      2200                                                      2250

CGCCCGACGTCTTTGCCGGACGAGTGGTGGACAGTGTGAATACCGCTGGGGAAAATTCCGCCCGTTTTTACTCTTCGGTACTGCGGCCGTTAATGATCTTCAGCGTGCTGGTATTCTGGGTGC
                    2300                                                  2350

TGACCGACTGGAGCCATGGTAGCAAAGTGGTGTATGCATG
2400                                  2439
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FIG 2C

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FIG 3

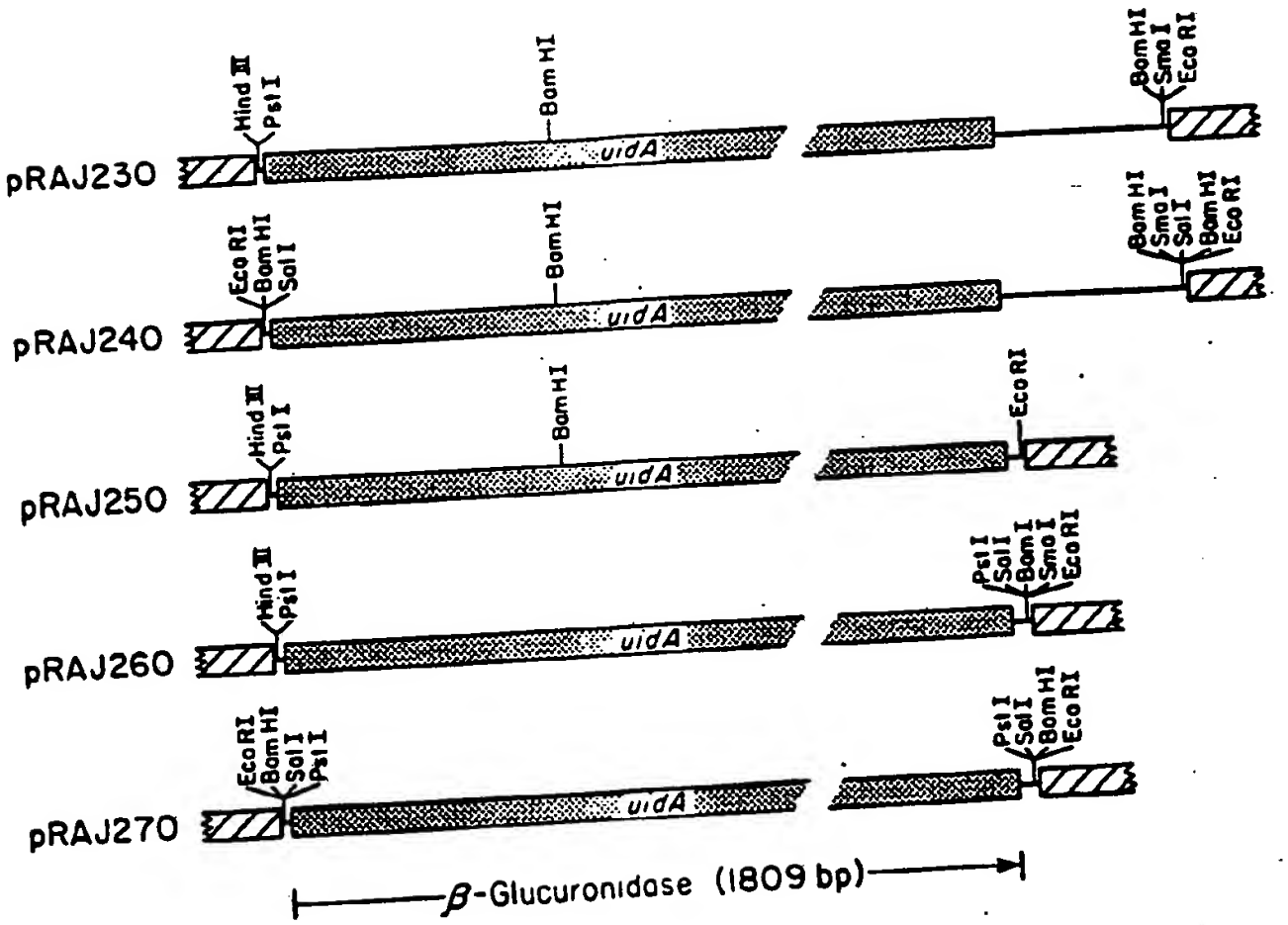

FIG 4

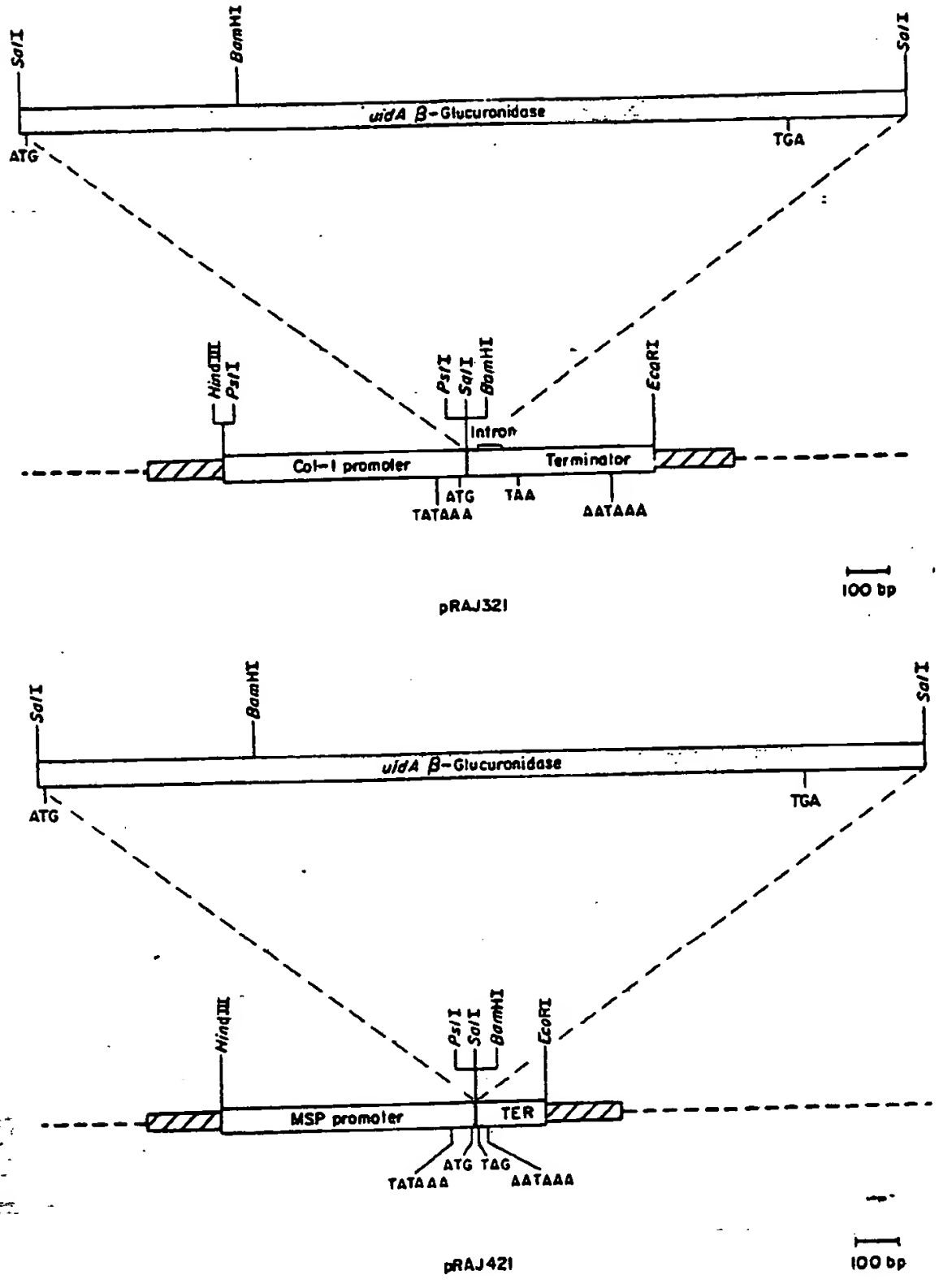
SalI
BamHI
SalI
uidA β-Glucuronidase
ATG
TGA
HindIII
PstI
PstI
SalI
BamHI
EcoRI
Intron
Col-1 promoter
Terminator
ATG
TATAAA
TAA
AATAAA
100 bp
pRAJ321
SalI
BamHI
SalI
uidA β-Glucuronidase
ATG
TGA
HindIII
PstI
SalI
BamHI
EcoRI
MSP promoter
TER
ATG
TATAAA
TAG
AATAAA
pRAJ421
100 bp

FIG. 5

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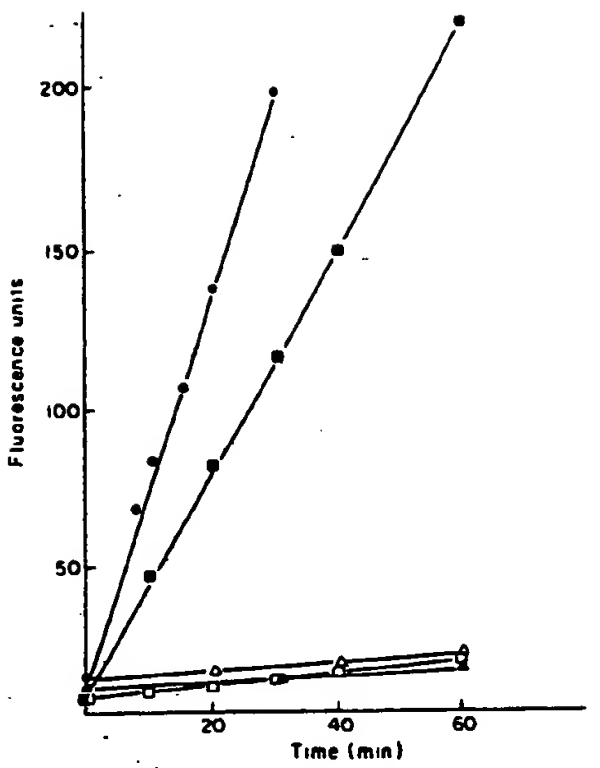

(●) DH408/pRAJ321 (in-frame *col-1 : uid-A*): (▲) DH408/pRAJ323 (out-of-frame *col-1 : uid-A*): (△) DH408 (untransformed): (■) DH408/pRAJ321 treated with pre-immune serum: (□) DH408/pRAJ321 treated with anti-β-glucuronidase antibody.

FIG 6

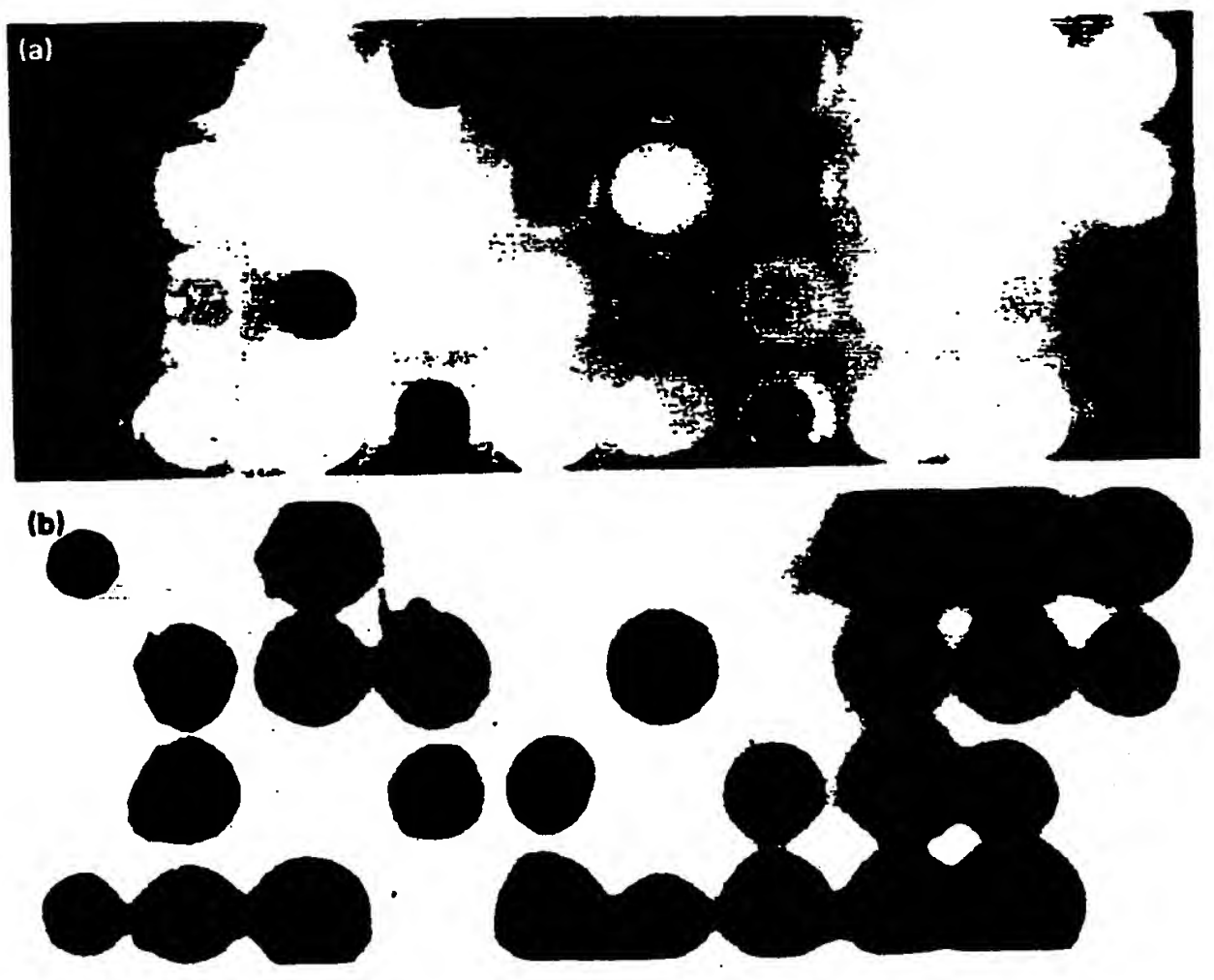

FIG 7

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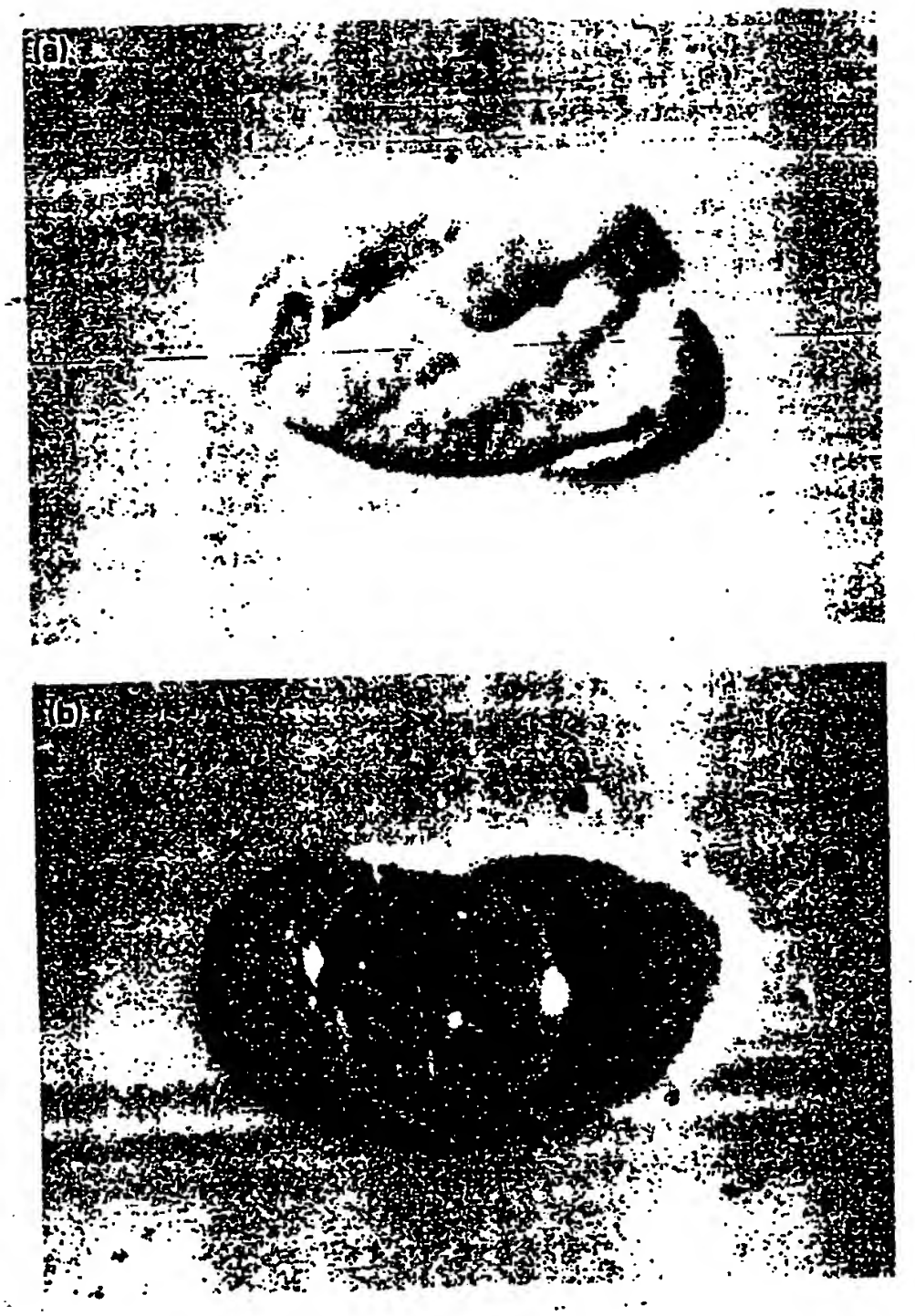
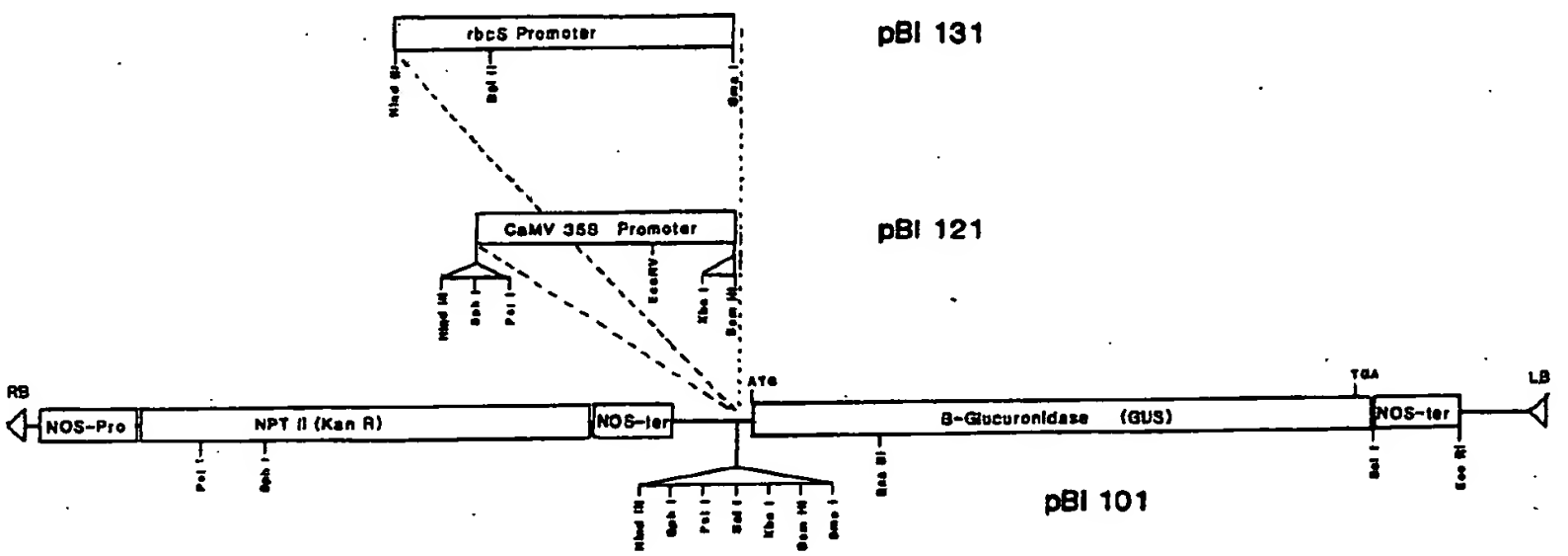


FIG 8



pBI101.1	Bam HI GGATCCCC G GGT GGT CAG TCC CTT ATG Sma I
pBI101.2	Bam HI GGATCCCC GG GTA GGT CAG TCC CTT ATG Sma I --
pBI101.3	Bam HI GGATCCCC GGG TAC GGT CAG TCC CTT ATG Sma I

FIG 9

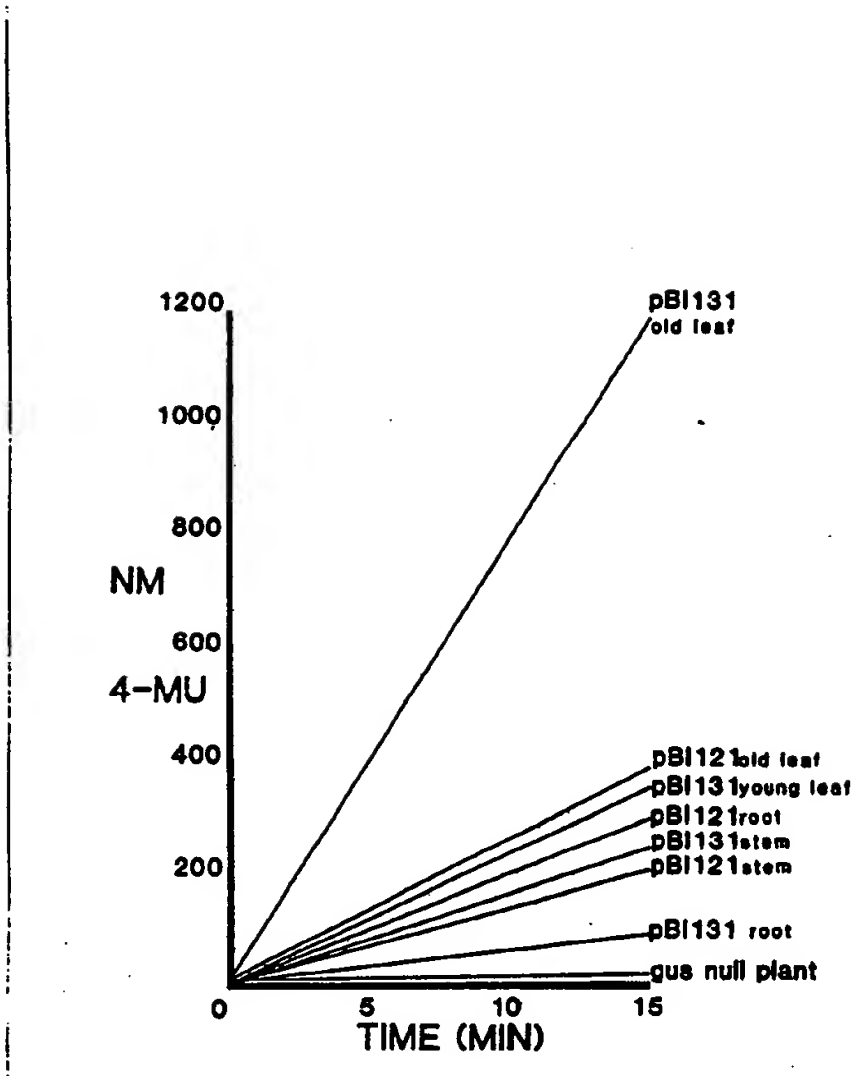

1200
1000
800
NM
600
4-MU
400
200
0
5
10
15
TIME (MIN)
pBI131 old leaf
pBI121 old leaf
pBI131 young leaf
pBI121 root
pBI131 stem
pBI121 stem
pBI131 root
gus null plant

FIG 10

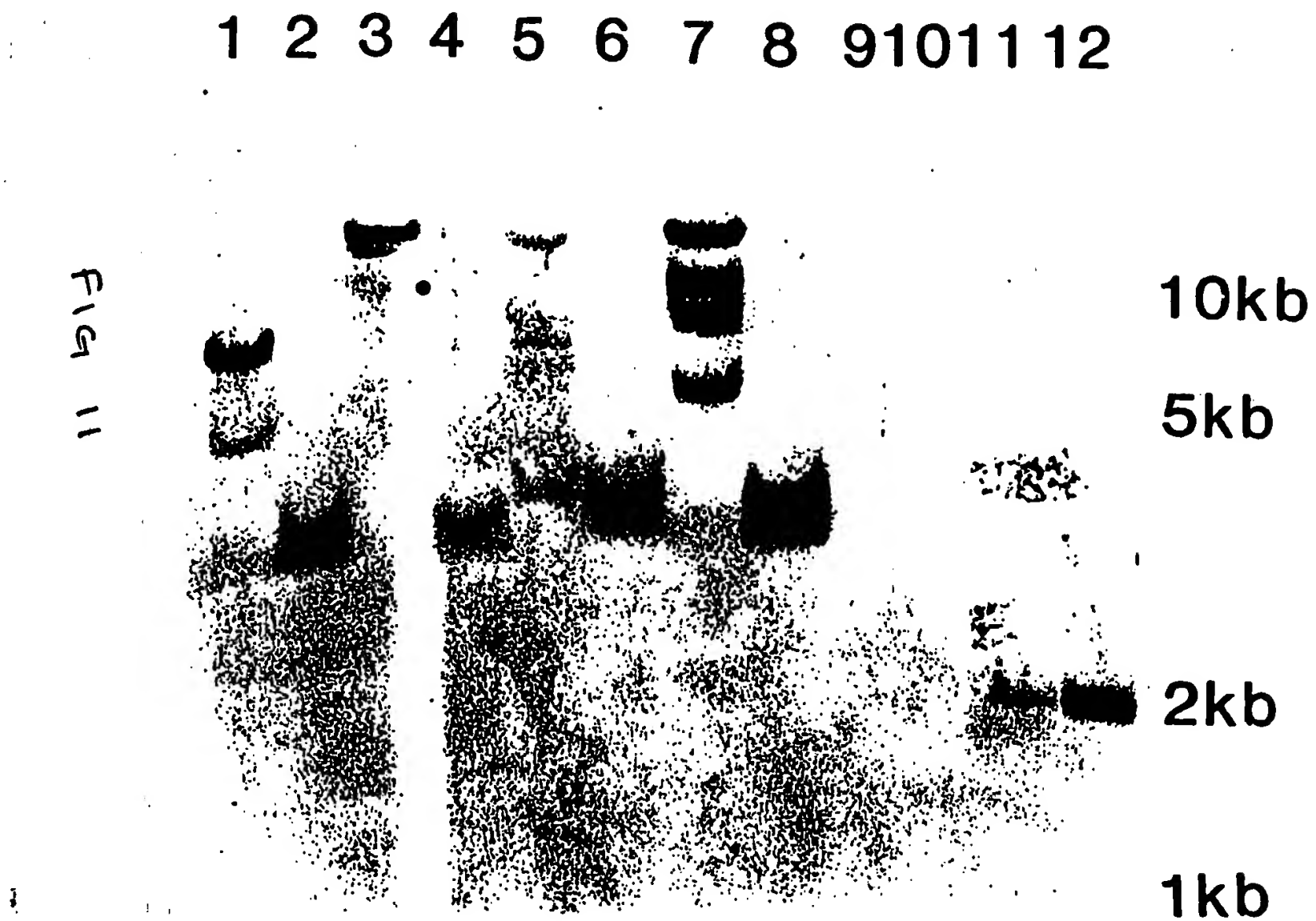


FIG 11/12

FIG 12

Gene Fusion

Field of invention

This invention relates to gene fusion and concerns novel gene fusion products and plasmids and the use thereof eg in monitoring gene expression.

Background to the invention

The use of fusions between a gene of interest and a reporter gene with an easily detectable product offers several advantages for the study of gene expression.

Control of gene activity can be manifested at many levels, including the initiation of transcription or translation, the processing, transport or degradation of mRNA or protein. The use of precise gene fusions can simplify analysis of this complex process and delineate the contribution of transcriptional control by eliminating the specific signals for post-transcriptional controls and replacing them with sequences from a readily asssayed reporter gene.

The use of a single set of assays to monitor the expression of diverse gene control regions simplifies analysis and often enhances the sensitivity with which measurements of gene activity can be made. Many genes in higher organisms are members of gene families consisting of several related genes whose expression may be

independently controlled (see reference 1). It is often desirable to study the expression of one member of such a gene family free from the background of the other members of the family. The use of *in vitro* generated gene fusions and DNA transformation permits such an analysis.

Further, members of multi-gene families whose products are very similar can be regulated differentially during development. By using gene fusions to individual members of such families and introducing these fusions into the germline, it is possible to study the expression of individual genes separate from the background of the other members of the gene family. Additionally, analysis of mutationally altered genes in organisms accessible to transformation techniques is greatly facilitated by the use of sensitive reporter enzymes. By using a reporter gene that encodes an enzyme activity not found in the organism being studied, the sensitivity with which chimaeric gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the available assays for the enzyme.

The most frequency used reporter gene is probably the *E. coli lacZ* gene, encoding a beta-galactosidase (see references 2, 3). Beta-galactosidase has many features that make it attractive as a gene fusion marker. The gene and gene product are well characterised genetically and biochemically (see reference 3). There are sensitive assays for the enzyme that utilise commercially available substrates, including several that allow visualisation of enzyme activity *in situ*. Beta-galactosidase is not, however, ideal for all systems. There are several intensively studied biological systems (eg tobacco plants) in which endogenous beta-galactosidase levels are high

enough for it to be difficult or impossible to detect chimaeric beta-galactosidase by enzymatic methods. In addition the enzyme and gene are very large, sometimes making the in vitro construction and analysis of gene fusions unwieldy.

Use of the Agrobacterium tumefaciens Ti-plasmid-encoded genes nopaline synthase (see references 4, 5) and octopine synthase (see reference 6) promised to overcome problems associated with endogenous activity as the opines produced by these genes are not found in normal plant cells. But because the assays are cumbersome and difficult to quantitate, cannot be used to demonstrate enzyme localisation (see reference 7), and octopine synthase cannot tolerate amino-terminal fusions (see reference 8) these reporter genes are not widely used. In addition the catabolism of opines by Agrobacterium often present in transformed plant tissue makes the interpretation of opine levels difficult in experiments where opine synthases are used as reporter genes (see reference 9).

The two most useful reporter genes have been the bacterial genes choloramphenicol acetyl transferase (CAT) and neomycin phophotransferase (NPTII) which encode enzymes not normally found in plant tissues (see references 10, 11, 12, 13). In addition NPTII can tolerate large amino-terminal fusions and remain enzymatically active, making it useful for studying organelle transport in plants (see reference 14). However both CAT and NPTII are relatively difficult, tedious and expensive to assay and suffer from variable endogenous activities in plant cells, which limits their sensitivity (see references 15, 16). In addition, competing reactions catalyzed by endogenous esterases, phosphatases, transferases and other enzymes

also limits sensitivity and makes quantitaion of CAT or NPTII by enzyme kinetics very difficult.

Recently, the firefly luciferase gene has been used as a marker in transgenic plants (see reference 60), but the enzyme is labile and difficult to assay with accuracy (see reference 61), the reaction is complex and there is little, if any, potential for routine histochemical analysis or fusion genetics.

Summary of the invention

The invention provides a new gene fusion system that uses a gene coding for beta-glucuronidase as the reporter gene.

Thus in one aspect the present invention provides a gene fusion product comprising a gene coding for beta-glucuronidase (GUS).

Beta-glucuronidase (GUS) is an acid hydrolase that catalyzes the cleavage of a wide variety of beta-glucuronides (see reference 36). Substrates for beta-glucuronidase are generally water soluble, and due to the extensive analysis of mammalian glucuronidases (see reference 17) many substrates are commercially available, including substrates for spectrophotometric, fluorometric and histochemical analyses. This ability to perform histochemical analysis of gene fusions is an important feature for the study of gene expression in metazoa and plants, where spatial discrimination is often essential for assessing the regulation of genes. Methods have been described that allow subcellular localisation of glucuronidase activity (reviewed in reference 18).

The invention may use a gene coding for GUS obtained from a wide variety of sources, including micro-organisms, animals such as mammals etc. It is currently preferred to use the uidA gene of E. coli for this purpose as most work so far has been carried out using this gene.

The uidA gene has been analyzed genetically and was shown by Novel et al to encode the beta-glucuronidase structural gene (see references 19, 20, 21, 22). The gene has now been fully sequenced and is found to encode a stable enzyme that has desirable properties for the construction and analysis of gene fusions.

The gene coding for GUS is conveniently under control of a suitable promoter, such as the E. coli lacZ promoter. For example, gene fusions of the E. coli lacZ promoter and coding region of the uidA gene have been constructed and show GUS activity under lac control.

Gene fusions using the cauliflower mosaic virus (CaMV) 35S promoter or the promoter from the small subunit of ribulose bisphosphate carboxylase (rbcS) and the gene coding for GUS have also been constructed. With such products the promoters have been shown to direct expression of GUS in transformed plants.

In a further aspect, the invention also provides a plasmid comprising a gene coding for GUS.

Such a plasmid may conveniently be constructed from plasmids such as pBIN19. A range of such plasmids may be produced, including those known as pBI101, including pBI101.1 (previously known as pTAK1) (Deposited with

National Collection of Industrial Bacteria (NCIB) under accession No. 12353), pBI101.2 (previously known as pTAK2) (NCIB accession No. 12354), and pBI101.3 (previously known as pTAK3) (NCIB accession No. 12355).

The pBI101 plasmids contain many different restriction sites (eg for Hind III, Sal I, Xba 1, BamH 1 and Sma I) upstream of the AUG initiator code of GUS, to which promoter DNA fragments can be conveniently ligated. For example the CaMV 35S promoter may be ligated into the Hind III and BamH 1 sites to create a plasmid known as pBI121 (previously known as pCTAK1). Similarly, the promoter from a tobacco ribulosebisphosphate carboxylase small subunit gene, d23 deleted of rbcS coding sequences fused to pBI101.1 makes a plasmid known as pBI131 (previously known as pSSU8TAK1).

Plasmids in accordance with the invention, particularly the pBI101 and related plasmids, are suitable for transcriptional or translational fusions, the latter possibility permitting additional proteins to be produced in a host whilst retaining enzyme activity of GUS.

The 3 pBI101 vectors differ by 1 or 2 initial nucleotides, permitting protein fusions to be constructed in all 3 reading frames.

The pBI101 and related plasmids incorporate the GUS coding sequence in a format that is easy to manipulate and transfer into hosts, particularly plants.

The products of the invention can be introduced to hosts such as plants, animals, yeasts, etc and are useful for monitoring and manipulating gene expression in such

recipients.

Hence in a further aspect, the invention provides a method of introducing a gene of interest to a host, comprising introducing to the host a gene fusion product comprising a gene coding for GUS and the gene of interest.

The invention can thus be used to introduce useful genes to a host eg confering desirable properties such as disease resistance etc on the host.

The present invention also provides a method of monitoring expression of a gene of interest in a host, comprising introducing to a host a gene fusion product comprising a gene coding for GUS and the gene of interest, and monitoring to detect the presence of GUS.

Expression of the gene of interest also results in expression of GUS. Provided no other source of GUS is present, the presence of GUS indicates expression of the gene of interest.

The monotiring may be carried out qualitatively or quantitively as appropriate, eg using spectrophotometric, fluorometric or histochemical assays.

Possible hosts include plants, animals, yeasts or other micro-organisms, and assays can be carried out both in vivo and in vitro.

The invention also includes within its scope a transformed host to which has been introduced a gene fusion product comprising a gene coding for GUS.

Products of such a transformed host, eq seeds of a transformed plant, are also included within the scope of the invention.

Gene fusion products in accordance with the invention may also be used as genetic markers in conventional plant breeding techniques such as restriction fragment length polymorphism (RFLP) techniques.

GUS is a highly suitable enzyme for use in study of gene expression and use of GUS gene fusions can allow analysis of genes whose products are of moderate and low abundance. Activity of the reporter enzyme is maintained when fused to other proteins at its amino terminus, allowing the study of translation and the processing events involved in protein transport. The reporter enzyme is detectable with sensitive histochemical assays, enabling localisation of gene activity in particular cell types. Finally, the reaction catalyzed by the reporter enzyme is sufficiently specific to minimize interference with normal cellular metabolism and general enough to allow the use of a variety of novel substrates to maximise the potential for fusion genetics and in vivo analysis.

The GUS gene has several features that make it particularly useful as a reporter gene for plant studies.

All plants thus far assayed lack detectable glucuronidase activity, providing a null background in which to assay chimaeric gene expression. Further, the enzyme is easily, sensitively and cheaply assayed both in vitro and in gels, and can also be assayed histochemically to localise GUS activity in cells and tissues. For example, it has been found that a fluorometric GUS assay can cost as little as

1/2000 of the cost of a comparable CAT assay, while giving increased sensitivity.

Expression of GUS can be accurately measured using fluorometric assays of trace amounts of transformed plant tissue. Plants expressing GUS are normal, healthy and fertile. GUS is very stable, retaining activity after electrophoresis on SDS polyacrylamide gels, and extracts continue to show high levels of glucuronidase activity after prolonged storage.

The invention will be further described, by way of example, with reference to the accompanying drawings, in which:

Figure 1 illustrates subcloning and strategy for determining the nucleotide sequence of the uidA gene;

Figure 2 illustrates the DNA sequence of the 2439 bp insert of pRAJ220, containing the beta-glucuronidase gene;

Figure 3 illustrates GUS gene module vectors;

Figure 4 illustrates the results of 7.5% SDS-PAGE analysis of beta-glucuronidase;

Figure 5 illustrates structures of the col-1:GUS fusion (pRAJ321) and the MSP:GUS fusion (pRAJ421);

Figure 6 shows the results of assaying beta-glucuronidase activity in transformed worms;

Figure 7 illustrates the results of co-segregation

analysis of beta-glucuronidase activity and transforming DNA;

Figure 8 illustrates histochemical visualisation of beta-glucuronidase activity in worms transformed with the col-1:GUS fusion (pRAJ321);

Figure 9 illustrates the structure of expression vectors;

Figure 10 is a graph illustrating beta-glucuronidase activity in extracts of different organs of transformed and non-transformed tobacco plants;

Figure 11 is an autoradiograph of a Southern blot of DNA extracted from transformed plants and digested with restriction endonucleases; and

Figure 12 illustrates 7.5% SDS-polyacrylamide gel stained for beta-glucuronidase activity.

Materials and Methods

DNA Manipulation

Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs whenever possible, and used as per the instructions of the manufacturer. Plasmid DNA preparations were done by the method of Birnboim and Doly (see reference 25) as described in the Maniatis et al (see reference 26). Routine cloning procedures, including ligations and transformation of E. Coli cells, were performed essentially as described in reference 26. DNA fragments were purified from agarose gels by electrophoresis onto Schleicher & Schuell NA 45 DEAE

membrane (see reference 27) as recommended by the manufacturer. DNA sequences were determined by the dideoxy chain terminator method of Sanger and Coulson (see reference 28) as modified by Biggin et al (see reference 29). Oligonucleotide primers for sequencing and site-directed mutagenesis were synthesised using an Applied Biosystems DNA synthesiser, and purified by preparative polyacrylamide gel electrophoresis. Site directed mutagenesis was performed on ssDNA obtained from pEMBL derived plasmids, essentially as described in reference 30. The strain used for routine manipulation of the uidA gene was RJ21, a recA derivative of JM83 (see reference 31) generated by P1 transduction. Strain PK803 was obtained from P. Keumpel of the University of Colorado at Boulder, and contains a deletion of the manA - uidA region. Plasmid vectors pUC7, 8 and 9 (see reference 11) and pEMBL (see reference 32) have been described.

Protein sequencing and amino acid analysis

Sequence analysis was performed by Dr A Smith of the Protein Structure Laboratory, University of California, Davis, using a Beckman 890M spinning-cup sequenator. Amino acid composition was determined by analysis of acid hydrolyzates of purified beta-glucuronidase on a Beckman 6300 amino acid analyser.

Protein analysis

Protein concentrations were determined by the dye-binding method of Bradford (see reference 33) using a kit supplied by BIO-RAD Laboratories. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli system (see reference 34).

Beta-glucuronidase assays

Glucuronidase was assayed in a buffer consisting of 50 mM $NaPO_4$, pH 7.0, 10mM B-ME, 0.1% Triton X-100, 1 mM p-nitrophenyl beta-D-glucuronide. Reactions were performed in one ml. volumes at 37°C, and terminated by the addition of 0.4 ml 2.5 M 2-amino-2-methyl propanediol. p-nitrophenol absorbance was measured at 415 nm. Routine testing of bacterial colonies for beta-glucuronidase activity was done by transfering bacteria with a toothpick into microtiter wells containing the assay buffer. The histochemical substrate 5-bromo. 4-chloro, 3-indolyl beta-D-glucuronide (analogous to the beta-galactosidase substrate X-gal) is commercially available (Research Organics Inc. Cleveland, Ohio), and is found to be an excellent and sensitive indicator of beta-glucuronidase activity *in situ* when included in agar plates at a concentration of 50 ug/ml.

Purification of beta-glucuronidase

Beta-glucuronidase was purified by conventional methods from the strain RJ21 containing the plasmid pRAJ210. Figure 1 illustrates subcloning and strategy for determining the nucleotide sequence of the *uidA* gene. Briefly, pBK*uidA* was generated by cloning into pBR325. pRAJ210 and pRAJ220 were generated in pUC9, with the orientation of the *uidA* gene opposite to that of the *lacZ* gene in the vector. The sequence was determined from both strands for all of the region indicated except from nucleotide 1 or 125. The orientation of the coding region is from left to right.

Results

Subcloning and Sequencing of the uidA gene

The starting point for the subcloning and sequencing of the beta-glucuronidase gene was the plasmid pBK*uidA* shown in Figure 1. pBK*uidA* has been shown to complement a deletion of the *uidA* - *manA* region of the *E. coli* K-12 chromosome restoring beta-glucuronidase activity when transformed into the deleted strain, PK803. The strategy for the localisation of the gene on the insert is shown in Figure 1.

A restriction map of the insert was obtained, and various subclones were generated in the plasmid vector pUC9, and tested for their ability to confer beta-glucuronidase activity upon transformation of PK803. The intermediate plasmid pRAJ210 conferred high levels of glucuronidase activity on the deleted strain, and was used for the purification of the enzyme. Several overlapping subclones contained within an 800 base pair (bp) *Eco RI* - *Bam HI* fragment conferred high levels of constitutive beta-glucuronidase production only when transformed into a *uidA*$^+$ host strain, and showed no effect when transformed into PK803.

It is surmised that the 800 bp fragment carried the operator region of the *uidA* locus, and was possibly titrating repressor to give a constitutively expressing chromasomal *uidA*$^+$ gene. With this information to indicate a probable direction of transcription, and a minimum gene size estimate obtained from characterisation of the purified enzyme (see below), a series of BAL 31 deletions were generated from the *Xho* 1 site of pRAJ210. The

fragments were gel purified, ligated into pUC9 and transformed into PK803. The resulting colonies were then assayed for beta-glucuronidase activity.

The smallest clone obtained that still gave constitutive levels of beta-glucuronidase was pRAJ220, which contained a 2.4 kilobase pair (kb) insert. Subclones of this 2.4 kb fragment were generated in M13mp8 and mp9 and their DNA sequence was determined as illustrated in Figure 2.

Figure 2 shows the DNA sequence of the 2439 bp insert of pRAJ220, containing the beta-glucuronidase gene. The arrows before the coding sequence indicate regions of dyad symmetry that could be recognition sequences for effector molecules. The overlined region is the putative Shine/Dalgarno sequence for the uidA gene, while the brackets indicate two possible Pribnow boxes. All of the palindromic regions fall within the smallest subcloned region (from the Sau 3A site at 166 to the Hinf I site at 291) that gave constitutive genomic expression of uidA when present in high copy in trans, consistent with their proposed function as repressor binding sites. The terminator codon at 2106 overlaps with an ATG that may be the initiator codon of a second open reading frame, as indicated (see Discussion).

Manipulation of the uidA gene for vector construction

The plasmid pRAJ220 contains the promoter and operator of the E coli uidA locus, as well as additional out-of-frame ATG codons that would reduce the efficiency of proper translational initiation in eukaryotic systems (see reference 35). It was necesary to remove this DNA to facilitiate using the structural gene as a reporter module

in gene fusion experiments. This was done by cloning and manipulating the 5' region of the gene separately from the 3' region, then rejoining the to parts as a lacZ:uidA fusion that showed beta-glucuronidase activity under lac control. The resulting plasmid was further modified by progressive subcloning, linker additions and site-directed mutagenesis to generate a set of useful gene module vectors. These manipulations are illustrated by reference to Figure 3, which shows GUS gene module vectors.

As illustrated in Figure 3, pRAJ220 (see Figure 1) was digested with Hinf I, which cleaves between the Shine/Dalgarno sequence and the initiator ATG, the single-stranded tails were filled-in, digested with Bam HI and the resulting 515 bp fragment was gel purified and cloned into pUC9/Hinc II and Bam HI. This plasmid was digested with Bam HI and the 3' region of the uidA gene carried on a 1.6 kb Bam HI fragment from pRAJ220 was ligated into it. The resulting plasmid, pRAJ230, showed IPTG inducible GUS activity when transformed into JM103. pRAJ230 was further modified by the addition of Sal 1 linkers to generate pRAJ240, an in-frame lacZ:uidA fusion in pUC7. pRAJ230 was digested with Aat II, which cuts 45 bp 3' to the uidA translational terminator, the ends were filled, digested with Pst I, and the resulting 1860 bp fragment was gel purified, and cloned into pEMBL9/Pst I and Sma I. The resulting plasmid, pRAJ250, is an in-frame lacZ:uidA fusion. The Bam HI site that occurs within the coding region at nucleotide 807 was eliminated by oligonucleotide-directed mutagenesis of single-stranded DNA prepared from pRAJ250, changing the Bam HI site from GGATCC to GAATCC, with no change in the predicted amino acid sequence. The clone resulting from the mutagenesis, pRAJ255, shows normal GUS activity, and lacks the Bam HI

site. This plasmid was further modified by the addition of a Pst I linker to the 3' end and cloned into pEMBL9/Pst I, to generate pRAJ260.

Purification and properties of beta-glucuronidase

Beta-glucuronidase activity in E. coli is induced by a variety of beta-glucuronides; methyl glucuronide is among the most effective (see reference 36). To determine the size and properties of the enzyme and to verify that the enzyme produced by the clone pRAJ210 was in fact the product of the uidA locus, the protein was purified from the over-producing strain, and the purified product was compared with the enzyme induced from the single genomic locus by methyl glucuronide.

Aliquots of supernatants from induced and uninduced cultures of E. coli C600 were analysed by SDS-PAGE and compared with aliquots of the purified beta-glucuronidase as shown in Figure 4. In Figure 4 lane (a) is molecular weight standards; lane (b) is extract from uninduced C600; lane (c) is extract from C600 induced for beta-glucuronidase with MeGlcU (see reference 23); lane (d) is 0.3 ug of purified beta-glucuronidase (calculated to contain the same activity as the induced extract); lane (e) is 3.0 ug aliquot of purified beta-glucuronidase.

The induced culture of C600 shows only a single band difference relative to the uninduced culture. The new band co-migrates with the purified beta-glucuronidase, indicating that the enzyme purified from the over producing plasmid has the same subunit molecular weight as the wild-type enzyme.

The purified enzyme was analysed for amino acid composition and subject to eleven cycles of Edman degradation to determine the amino terminal sequence of amnio acids. The amino acid composition agrees with the predicted composition derived from the DNA sequence, and the determined amino acid sequence agrees with the predicted sequence, identifying the site of translational initiation and indicating that the mature enzyme is not processed at the amino terminus.

E. coli beta-glucuronidase is a very stable enzyme, with a broad pH optimum (from pH 5.0 to 7.5); it is half as active at pH 4.3 and pH 8.5 as at its neutral optimum, and it is resistant to thermal inactivation at 50°C.

Discussion

Molecular analysis of the uid locus

The complete nucleotide sequence of the E. coli uidA gene, encoding beta-glucuronidase, has been determined. The coding region of the gene is 1809 bp long, giving a predicted subunit molecular weight for the enzyme of 68,200 daltons, in agreement with the experimentally determined value of about 73,000 daltons. The translational initiation site was verified by direct amino acid sequence analysis of the purified enzyme.

Genetic analysis of the uidA locus has shown three distinct controlling mechanisms, two repressors and a cAMP dependent factor, presumably CAP (see reference 22). The DNA sequence determined includes three striking regions of dyad symmetry that could be the binding sites for the two repressors and the CAP protein. One of the sequences

matches well with the consensus sequence for CAP binding, and is located at the same distance from the putative transcriptional intitiation point as the CAP binding site of the lac promoter. It is interesting that the putative CAP binding site overlaps one of the other palindromic sequences, suggesting a possible antagonisitc effect of CAP and one or both repressors.

The sequence analysis indicates the presence of a second open reading frame of at least 340 bp, whose initiator codon overlaps the translational teminator of the uidA gene. This open reading frame is translationally active. Although a specific glucuronide permease has been described biochemically (see reference 36), the level of genetic analysis performed on the uid locus would not have distinguished a mutation that eliminated glucuronidase function from a mutation that eliminated transport of the substrate (see refences 19, 20). All mutations that specifically eliminated the ability to grow on a glucuronide mapped to the uidA region of the E. coli map, indicating that if there is a gene responsible for the transport of glucuronides, it is tightly linked to uidA. By analogy to the lac operon, it is proposed that the coupled open reading frame may encode a permease that facilitates the uptake of beta-glucuronides.

The uidA gene as gene fusion marker

Plasmid vectors have been constructed in which the uidA structural gene has been separated from its promoter/operator and Shine/Dalgarno region, and placed within a variety of convenient restriction sites. The GUS gene on these restriction fragments contains all of the beta-glucuronidase coding information, including the

initiator codon; there are no ATGs upstream of the initiator. These vectors allow the routine transfer of the beta-glucuronidase structural gene to the control of heterologous sequences, thereby facilitating the study of chimaeric gene expression in other systems.

The uidA encoded beta-glucuronidase is functional with several combinations of up to 20 amino acids derived from the lacZ gene and/or polylinker sequences. Translational fusions to GUS have also been used successfully in transformation experiments in the nematode Caenorhabditis elegans, and in Nicotiana tabacum, giving enzyme activity with many different combinations of amino terminal structures (see below).

There are several systems currently amenable to DNA transformation in which the study of gene fusions using beta-glucuronidase as the reporter enzyme may be advantageous. Very little, if any, beta-glucuronidase activity has been detected in most higher plants, including tobacco (Nicotiana tabacum), potato (Solanum tuberosum), and soybean (Glycine max). Fusions of beta-glucuronidase to several plant genes have recently been used to monitor tissue-specific gene activity in transformed tobacco plants. There is no detectable beta-glucuronidase activity in the slime mold Dictyostelium discoidum or the yeast Saccharomyces cerevisiae. Extracts from Drosophila melanogaster have shown no beta-glucuronidase activity under conditions that show beta-galactosidase levels several hundred-fold over background.

Experiments have also been performed which show the expression of transformed genes in the nematode

Caenorhabditis elgans using the gene fusion system. Briefly, vectors consisting of the flanking regions of a collagen gene (col-1) or a major sperm protein gene of C. elegans fused to the Escherichia coli uidA gene, encoding beta-glucuronidase, were microinjected into worms and found to be propagated as high-copy extrachromosomal tandem arrays. Beta-glucuronidase activity was detected in transformed lines, and the activity has been shown to be dependent upon the correct reading frame of the construction and on the presence of the worm sequences. The enzyme activity was shown to be encoded by the chimaeric beta-glucuronidase gene by co-segregation analysis and by inactivation with specific antisera. Expression is at a very low level, and seems to be constitutive. Histochemical techniques have been used to visualise the enzyme activity in embryos.

In particular, gene fusions between GUS and two well-characterised genes of C. elegans, col-1 and MSP (p3L4) have been constructed. col-1 is a collagen gene that is transcribed predominantly in embryos, and somewhat less in later developmental stages, and presumably encodes a component of the first larval stage cuticle (see references 37, 38).

The MSP gene P3L4 is a transcribed member of the major sperm protein gene family, which encodes a set of abundant, closely related 15,000 M proteins that are present only in sperm (see references 39, 40). The MSP genes are transcribed only during spermatogenesis, which occurs during the fourth larval stage (L4) in hermaphrodites and in L4 and adult stages in males.

Results and Discussion

The structures of the in-frame col-1:GUS fusion pRAJ321 and the in-frame MSP:GUS fusion pRAJ421 are shown in Figure 5. In Figure 5 both vectors are built within pUC9 (see reference 31). The hatched region is the lac-derived sequence from pUC9. pRAJ321 is 5.8 kb. pRAJ421 is 5.5 kb. DNA manipulations were performed essentially as described in reference 26. pRAJ321 encodes the first 5 amino acids of the col-1 gene product plus 9 amino acids derived from linker sequences fused in-frame to the entire coding region of the E. coli uidA gene, and followed by the 3' intron, the translational termination codon and the polyadenylation signal from the col-1 gene. The col-1 promoter module extends from the Hinc II site 530 bp upstream from the transcription initiation site to a BAL31 generated breakpoint 14 bp into the protein coding sequence of col-1. This fragment cloned into the Hind III and Pst I sites of pUC9 is designated pRAJ301. pRAJ303 was generated by elimination of the promoter-proximal Pst site of pRAJ 301, and insertion of an octameric Pst I linker. The 3' intron and the polyadenylation site from the col-1 gene are contained on a 436 bp Pvu II-Hind III fragment cloned into the Sma I site of pUC9, designated pRAJ310. The 570 bp Hind III-Sal I fragment from pRAJ301 and pRAJ303 were cloned into pRAJ310 to generate the expression vectors, pRAJ311, and pRAJ313. The MSP:GUS fusion vector pRAJ421, encodes the initiator methionine of the MSP coding sequence, 9 amino acids derived from linker sequences, and the GUS coding region, followed by the translational terminator of the MSP gene and the polyadenylation signal. The promotor module extends from a Hind III site 584 bp upstream from the initiator ATG to a Hind III site 4 bp into the coding sequence. Using a Pst I linker, this fragment was cloned into the Hind III-

Pst I sites of pUC9 and is designated pRAJ401. The MSP terminator module extends from the RsaI site located 8 nucleotides upstream from the translation terminator codon, to the Hind III site, a total of about 150 bp. This fragment was shown to contain the polyadenylation site. After several manipulations, the resulting 160 bp fragment was cloned into pRAJ401 that had been digested with Bam HI and Sma I. A plasmid clone was obtained that contained the terminator in the correct orientation to the promoter, designated pRAJ411. The uidA structural gene encoding beta-glucuronidase (GUS) was transferred into the expression vectors as a 2.1 kb Sal I fragment from the plasmid pRAJ240. Clones in the correct orientation were obtained, and the nucleotide sequences at the junctions were determined using specific oligonecleotide primers complementary to the 5' coding region of the uidA gene. pRAJ421 and pRAJ321 were shown to have GUS in-frame to the MSP and col-1 initiators, respectively, while GUS in pRAJ323 was shown to be out-of-frame with respect to the col-1 initiator.

Plasmid DNA was injected into the distal gonal arm of adult hermaphrodite worms at a concentration of approximately 500 ug/ml, essentially as described in reference 41. The strain used was DH408, lacking glucuronidase activity (see reference 46). Lines carrying the injected DNA as high-copy extrachromosomal tandem arrays were obtained from the F2 generation of the injected worms. Stability and physical properties of the tandem arrays were similar to those described in reference 41. Transformants were obtained containing either an in-frame col-1:GUS fusion (pRAJ321), an out-of-frame col-1:GUS fusion (pRAJ323), an in-frame MSP:GUS fusion (pRAJ421) or a GUS-encoding plasmid containing no worm

sequences (pRAJ210).

Fluorometric assays were performed in 100 ul of 50 mM-$NaPO_4$ (pH7.0), 10mM beta-mercaptoethanol, 0.1% (v/v) Triton X-100, 0.5mM-4-methyl umbelliferyl beta-D-glucuronide, at 37°C, and terminated with the addition of 1 ml of 0.2 M-Na_2CO_3, or 100 ul of 1 M-Na_2CO_3 (for small-scale, qualitative assays). The reaction products were visualised by irradiation with 365 nm light (for qualitative assays) or by fluorescence measurements with ex^{365nm} and em^{455nm}. Extracts were prepared from worms harvested from Petri plates, washed twice in M9 salts, resuspended in assay buffer without substrate and passed through a French pressure cell at 12,000lb/in^2. Protein concentrations were adjusted to 20 mg/ml and 50 ul portions were assayed. Measurements were made on a Perkin-Elmer LS-3 spectrofluorometer. Worms from the populations harvested were shown to contain the transforming DNA at similar copy number. Extracts prepared as described above were incubated with equal volumes of either neat pre-immune serum, or affinity-purified antibody directed against purified E. coli beta-glucuronidase, at a concentration of 125 ug/ml for 3 h at 4°C. Protein A-Sepharose was added, and the reactions were allowed to sit for 2 h at 4°C. The extracts were centrifuged at 12,000g for 5 minutes, and then assayed for beta-glucuronidase. Worms were grown for enzyme assays on the E. coli strain PK803 (obtained from P Kuempel) that contains a deletion of the uidA locus and has no detectable glucuronidase activity.

Extracts were prepared from populations of transformed worms and assayed for the presence of beta-glucuronidase.

The results are shown in Figure 6.

Extracts from uninjected worms, worms carrying the out-of-frame col-1:GUS fusion, or pRAJ210 showed no detectable beta-glucuronidase activity, while extracts from worms carrying either the in-frame col-1:GUS fusion or the MSP:GUS fusion showed significant levels of enzyme activity. Reconstruction experiments with purified beta-glucuronidase in worm extracts indicated that the quantities of enzyme in the transformed extracts were about 1 to 2ng beta-glucuronidase/mg soluble protein, assuming comparable turnover numbers of the native and chimeric enzymes (data not shown). Consistent with the extremely low enzyme levels, no transcript was detected by Northern blot analysis, nor has an immunologically cross-reactive protein been detected in extracts, as judged by Western blots, to a sensitivity of about one part in 10^5 (data not shown).

To verify that the beta-glucuronidase activity measured in the extracts was due to the E. coli uidA gene product, portions of the extracts were incubated with antibody to homogeneous E. coli beta-glucuronidase or with pre-immune serum. Immune complexes were precipitated with Staph A-Sepharose, and the supernantant was assayed for beta-glucuronidase activity (Figure 6). Beta-glucuronidase activity diminshed only after addition of antibody directed against purified bacterial glucuronidase; pre-immune serum showed no effect.

It was possible that a small number of integrated copies of the chimaeric genes was responsible for the observed enzyme activity, and that the large tandem array was inactive. To test this possibility, 40 F2 worms from a

transformed individual were cloned and grown to saturation on plates. Extracts were prepared and assayed for the presence of the transforming DNA by dot blot hydridisation, and for beta-glucuronidase activity.

The 40 F2 worms derived from a transformant carrying the col-1:GUS fusion were cloned onto individual Petri plates, grown to saturation, harvested and washed, and the culture was split into 2 parts. Extracts were prepared for (a) fluorogenic beta-glucuronidase assays or (b) DNA dot blots. Glucuronidase assays were performed essentially as described in connection with Figure 6, in the wells of a Gilson tube rack at 37°C for 4 h, and visualised by addition of Na_2CO_3 and placing the rack on a long-wave-length ultraviolet light box. DNA dot blots were probed with ^{32}P-labelled pRAJ210. All worm cultures that gave rise to a positive signal by DNA dot blot analysis also gave rise to glucuronidase activity, and vice versa. In several repeats of this experiment no case was observed in which strict co-segregation of the high-copy DNA and beta-glucuronidase activity was not maintained.

The results are shown in Figure 7.

The high-copy transforming DNA and the enzyme activity always co-segregated, indicating that the extrachromosomal tandem array was responsible for the beta-glucuronidase activity. Identical results were obtained from transformed populations carrying either the MSP:GUS or the col-1:GUS fusions.

To determine whether temporal regulation of the transforming DNA was occurring, extracts from staged populations of transformed worms were assayed for beta-

glucuronidase activity. For both the col-1:GUS fusion and the MSP:GUS fusion, specific activity of B-glucuronidase was highest in embryos, and decreased with developmental time (data not shown). The temporal pattern of expression of the col-1:GUS fusion is consistent with the available data on col-1 expression as determined by DNA dot blots in Nothern blots (see reference 38). However, the pattern is also consistent with a low level constitutive expression of the chimaeric gene when accounting for the near 100-fold increase in the protein content of the worm during development. In the case of the major sperm gene fusion, this temporal pattern of expression is inconsistent with the normal expression of MSP genes only during the L4 stage, but is consistent with constitutive expression of the chimaeric gene.

In order to visualise beta-glucuronidase activity in situ, embryos were prepared from a population of worms containing the in-frame col-1:GUS fusion and an untransformed control population, fixed and assayed histochemically for beta-glucuronidase activity.

Freeze-cracked, formaldehyde-fixed (3% (w/v) paraformaldehyde in phosphate buffer (pH 7) for 3 min on ice) embryos from DH408 (a) or DH408 containing pRAJ321 (b) were assayed for glucuronidase activity using naphthol-ASBI glucuronide for 6 h at 37°C, and post-coupled with freshly prepared hexazonium pararosanalin (see reference 43).

The results are shown in Figure 8.

In the transformed population (a), many embryos show the red precipitate characteristic of beta-glucuronidase

activity, while the untransformed population (b) never shows staining. The number of positives in a given transformed population, and the intensity of staining within a population, varies considerably. Larvae and adults from a transformed population assayed under similar conditions did not show detectable staining. Perhaps because of the low levels of beta-glucuronidase in the transformed populations it has not been possible to localise the activity spatially within the embryo, either histochemically or by indirect immunofluorescence or immunocytochemistry (data not shown). Under the fixation conditions and lengthy assay times used to obtain histochemical staining of the transformed embryos (due to the low levels of beta-glucuronidase), the diffusion of the product may be preventing discrete localisation, if indeed it is occurring.

In summary, the GUS fusion system has been used to measure chimaeric enzyme levels in transformed worms. The expression of GUS in the transformed lines is dependent upon the presence of worm promoters, and on the correct reading frame of the translational fusions used. The levels of expression in these transformants are very low, but easily measured using a fluorometric enzyme assay for beta-glucuronidase, corresponding to about one part in a million of the soluble protein in a worm extract. The transforming DNA is in the form of long extrachromosomal tandem arrays, a situation that certainly does not mimic the normal in vivo condition of the genes under study. Possibly the structure of the arrays imposes characteristic restrains on the expression of genes within them, perahaps due to chromatin structure or a peculiarity of conformation. Is is possible that integration of the transforming DNA will allow higher levels of expression.

Methods have recently been developed to allow integration of exogenous DNA in C. elegans (see reference 44). Integration of the vectors described here into the germline of the worm may allow resolution of whether the low level, and inappropriate developmental expression of the chimeric genes, is due to their extrachromosomal tandem-array structure or to some other feature of the constructions.

Experiments were also carried out to demonstrate applicability of the GUS gene fusion system to plants.

Materials and Methods

Nucleic Acid Manipulation

DNA manipulations were performed essentially as described in reference 26. Enzymes were obtained from New England Biolabs, Boehringer or BRL.

Plant Transformation and Regeneration

Binary vectors containing CaMV-GUS fusions and rbcS-GUS fusions in E. coli MC1022 were mobilised into Agrobacterium tumefaciens LBA4404 as described in reference 45. The integrity of the vector in Agrobacterium was verified by preparing DNA from Agrobacterium immediately before plant transformation using the boiling method (see reference 56). Leaf discs of Nicotiana tabacum, var. Samsun were transformed using the leaf disc method (see reference 46), and transformed plants were selected on MS medium (see reference 47) containing 100 ug/ml kanamycin. Plants were maintained in axenic culture on MS basal medium, 3% sucrose, 200 ug/ml

carbenicillin and 100 ug/ml kanamycin, at approximately 2000 lux, 18 hour day, 26°C.

Southern Blot Analysis

DNA was prepared from plants by phenol extraction and ethanol precipitation of plant homogenates, followed by RNAse digestion, phenol extraction and isopropanol precipitation. Extracts were prepared from axenic tobacco plants using approximately 100 mg fresh weight of tissue ground in 500 ul extraction buffer. Ten ul of extract was incubated at 37°C in 4 ml assay buffer and 1.0 ml aliquots were withdrawn at 0, 5, 10 and 15 minutes intervals and stopped by addition to 1 ml 0.2 M Na_2CO_3. The fluorescence of liberated 4-MU was determined as described. Old leaves were lower, full-expanded leaves approximately 5 cm long, while young leaves were approximately 5 mm long, and were dissected from the shoot apex. All samples we taken from the same plant (either CaMV-GUS 21, SSU GUS 2 or non-transformed) at the same time. DNA samples (10 ug) were digested with restriction endonucleases, electrophoresed in an 0.8% agarose gel and blotted onto nitrocellulose (see reference 26). Filters were hybridised with oligomer-primed, ^{32}P labelled GUS gene fragment (see reference 57) and then washed with 0.2X SSC at 65°C.

Substrates

Substrates included: 4-methyl umbelliferyl glucuronide (MUG) (Sigma M-9130), 5-bromo-4-chloro-3-indolyl beta-D-glucuronide (X-GLUC) (Research Organics Inc., 4353E. 49th St, Cleveland, Ohio, USA), resorufin glucuronide (ReG) (Molecular Probes Inc., 4849 Pitchford Ave, Eugene,

Oregon, USA).

Lysis Conditions

Tissues were lysed for assays into 50mM NaH_2PO_4 pH7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM beta-mercaptoethanol (extraction buffer) by freezing with liquid nitrogen and grinding with mortar and pestle with sand or glass beads. Disposable pestles that fit into Eppendorf tubes (Kontes Glass) proved useful for homogenizing small bits of tissue (eg leaf). Extracts can be stored at -70°C with no loss of activity for at least two months. Storage of extracts in this buffer at -20°C should be avoided, as it seems to inactivate the enzyme.

Spectrophotometric Assay

For samples containing reasonably large amounts of beta-glucuronidase activity, the colorometric assay can be used, monitoring the appearance of yellow colour with time. Turbidity of colour in the extract can severely limit sensitivity. For a 1 ml reaction volume: 50 mM $NaPO_4$ pH 7.0, 10 mM beta-mercaptoethanol 1mM EDTA, 1 mM p-nitrophenyl glucuronide, 0.1% Triton X-100. Incubate at 37°C. The reaction is terminated by the addition of 0.4 ml of 2-amino, 2-methyl propanediol (Sigma A-9754). Absorbance is measured at 415 nm against a substrate blank (or if turbudity of the extract is a problem, against a stopped blank reaction to which an identical amount of extract has been added). Under these conditions the molar extinction coefficient of p-nitrophenol is assumed to be 14,000, thus in the 1.4 ml final volume, an absorbance of 0.010 represents one nanomole of product produced. One unit is defined as the amount of enzyme that produces one

nanomole of product/minute at 37°C. This represents about 5 ng of pure beta-glucuronidase.

Fluorometric Assay

For a review of fluorescence techniques, see reference 48. The fluorogenic reaction is carried out in 1 mM 4-methyl-umbelliferyl glucuronide in extraction buffer with a reaction volume of 1 ml. The reaction is incubated at 37°C, and 200 ul aliquots are removed at zero time and at subsequent times and the reaction terminated with the addition of 0.8 ml 0.2 M Na_2CO_3. The addition of Na_2CO_3 serves the dual purposes of stopping the enzyme reaction and developing the fluorescence of MU, which is about seven times as intense at alkaline pH. Fluorescence is then measured with excitation at 365 nm, emission at 455 nm on a Kontron SFM 25 Spectrofluorimeter, with slit widths set at 10 nm. The resulting slope at MU fluoresence versus time can therefore be measured independently of the intrinsic fluorescence of the extract. The fluorimeter should be calibrated with freshly prepared 4-methyl umbelliferone (MU) standards of 100 nanomolar and 1 micromolar MU in the same buffers. Fluoresence is linear from nearly as low as the machine can measure (usually 1 nanomolar or less) up to 5 - 10 micromolar 4-methyl umbelliferone.

A convenient and sensitive qualitative assay can be

done by placing the tubes on a long-wave UV light box and observing the blue fluorescence. This assay can be scaled down easily to assay very small volumes (reaction volume 50 ul, terminated with 25 ul 1M Na_2CO_3) in microtiter dishes or Eppendorf tubes.

If the intrinsic fluorescence of the extract limits sensitivity, it is possible to use other fluorogenic substrates. In particular, resorufin glucuronide has a very high extinction coefficient and quantum efficiency, and its excitation (560nm) and emission (590nm) are conveniently in a range where plant tissue does not absorb or fluoresce heavily. In addition, it fluoresces maximally at neutral pH, making it unnecessary to stop the reaction.

Protein concentrations of plant extracts and of purified beta-glucuronidase were determined by the dye-binding method of Bradford (reference 33), with a kit supplied by BIO-RAD Laboratories.

DNA concentrations in extracts were determined by measuring the fluorescence enhancement of Hoechst 33258 dye as described in reference 58, with the calibrations performed by addition of lambda DNA standards to the extract to eliminate quenching artefacts.

<u>In situ localisation of GUS activity in SDS polyacrylamide gels</u>

Plant extracts (1-50 ul) were incubated with 2 volumes of SDS Sample buffer at room temperature for approximately 10 - 15 minutes and then electrophoresed on a 7.5% acrylamide SDS gel (see reference 34) overnight at 50 mA, or in a

mini-gel apparatus (BIO-RAD) for 45 minutes. The gel was then rinsed 4 times with gentle agitation, in 100 ml extraction buffer for a total of 2 hours, incubated on ice in assay buffer (containing MUG) for 30 minutes, then transferred to a glass plate at 37°C. After approximately 10 - 30 minutes at 37°C, depending on the sensitivity required, the gel was sprayed lightly with 0.2 M Na_2CO_3 and observed under long wavelength UV transillumination. Gels were photographed using a Kodak 2E Wratten filter. For maximum sensitivity and resolution, it is important to allow the reaction at 37°C to proceed without liquid on the surface of the gel because the product of the reaction is very soluble, and will diffuse.

Histochemical Assay

Sections were cut by hand from unfixed stems of plants grown in vitro essentially as described in reference 49, and fixed in 0.3% formaldehyde in 10mM MES pH 5.6, 0.3M mannitol for 45 minutes at room temperature, followed by several washes in 50mM NaH_2PO_4, pH 7.0. All fixatives and substrate solutions were introduced into interstices of sections with a brief (about 1 minute) vacuum infiltration.

A good review of histochemical techniques and the caveats to their utilisation and interpretation can be found in Pearse (see reference 18). Substrates for histochenical localisation include the indigogenic dye 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), and napthol ASBI glucuronide.

Histochemical reactions with the indigogenic substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) were

performed with 1 mM substrate in 50mM NaH_2PO_4 pH 7.0 at 37°C for times from 20 minutes to several hours. After staining, sections were rinsed in 70% ethanol for 5 minutes, then mounted for microscopy.

Cleavage of napthol ASBI glucuronide releases the very insoluble free napthol ASBI which is either simultaneously coupled, or post-coupled with a diazo dye to give a coloured product at the site of enzyme activity. Post-coupling is preferred, as it seems to give a much lower background. Sections were incubated in 0.1 m $NaPO_4$ pH 7.0 with 1 mM Napthol ASBI glucuronide in a moist chamber at 37°C for 15 minutes to 3 hours. The specimen is then washed in phosphate buffer and coupled using a fresh solution of diazotised dye in phosphate buffer. Post-coupling with a 1-3 mg/ml solution of Fast Garnet GBC in phosphate buffer, pH 7, gives a very nice result after as little as thirty seconds coupling, after which the section is washed and mounted for light microscopy.

Fixation conditions will vary with the tissue, and its permeability to the fixative. Glutaraldehyde does not easily penetrate leaf cuticle, but works well with stem cross sections. Fixation with 2.5% glutaraldehyde in 0.1 M $NaPO_4$ pH 7.0 for 2-3 minutes on ice leaves a reasonable amount of GUS activity, when followed by extensive washing. 3% formaldehyde in 50 mM $NaPO_4$ pH 7.0 for 30 minutes on ice also works well, and penetrates leaf cuticle, leaving high GUS activity. It is recommended that fixation is tested empirically in any new system.

Purification of beta-glucuronidase

Beta-glucuronidase was purified essentially as described

from E. coli cells containing the plasmid pRAJ210 which contains the entire beta-glucuronidase coding region and promoter/operator. Eight liters of cells were grown in L broth with 50 ug/ml ampicillin at 37°C with vigorous agitation. The cells were harvested as they approached saturation, washed in M9 salts, and resuspended in about 100 ml of 100 mM $NaPO_4$ pH 7.0 10 mM beta-mercaptoethanol, 50 mM NaCL, 0.2% Triton X-100 and 25 ug/ml phenylmethyl sulphonyl fluoride. The slurry was passed through a French pressure cell at 12,000 psi, and the resultant lysate was stirred on ice for 30 minutes. The lysate was spun at 10,000 x g for 30 minutes at 4°C, and the turbid supernatant was dialysed overnight against several changes of 50 mM Tris pH 7.6, 10 mM B-ME (buffer A). The dialysate was loaded onto a column of DEAE Sephacel (2.5 x 40 cm) equilibrated in the same buffer, at 4°C. The column was washed with loading buffer and eluted with a 500 ml linear gradient of NaCl (0 - 0.4 M). The combined peak fractions were concentrated in an Amicon ultrafiltration apparatus with a PM 30 membrane to a final volume of 27 ml. This volume was loaded onto a 500 ml (2.5 x 100 cm) Sephacryl S-200 gel filtration column, and eluted with buffer A plus 100 mM NaCl. Peak fractions were pooled and dialysed overnight against 20 mM NaOAc pH 5.0, at 4°C. A precipitate formed that was collected by centrifugation at 5000 x g for 20 minutes at 4°C. The resulting pellet was dissolved in buffer A, and both pellet and supernatant were assayed for beta-glucuronidase activity and analysed by SDS-PAGE. The supernatant contained the majority of the activity, and by gel analysis had lost nearly all of the contaminating protein, (greater than 95% purity as judged by Coomassie staining) obviating further chromatograpy. The purified enzyme was stored in GUS extraction buffer at 4°C. The final yield

was about 350 mg.

Results

Higher Plants contain no detectable beta-glucuronidase activity

Roots, stems and leaves were taken from wheat, tobacco, tomato, potato, Brassica napus and Arabidopsis thaliana, potato tubers and seed from wheat and tobacco were homogenised with GUS extraction buffer containing a variety of protease inhibitors such as PMSF and leupeptin. The plant extracts were incubated in a standard assay at 37°C for 4 to 16 hours, and the fluorescence of MU was measured. Endogenous activity was below the limits of detection. Extremely lengthy assays occasionaly gave low levels of MU fluorescence, but the kinetics of MU accumulation were consistent with a slow conversion of the glucuronide into another form, possibly a glucoside, that was subsequently cleaved by intrinsic glycosidases. Beta-galactosidase assays performed under similar conditions on tobacco and potato extracts were off-scale (at least 10,000 times higher than the minimal detectable signal) within 30 minutes. Reconstruction experiments were performed with purified GUS added to tobacco and potato extracts to demonstrate the ability of these extracts to support beta-glucuronidase activity (data not shown).

Construction of plasmids for transformation of plants with GUS fusions

A general purpose vector for constructing gene fusions was made by ligating the coding region of GUS 5' of the nopaline synthase polyadenylation site (see reference 12)

in the polylinker sites of pBIN 19 (see reference 45). This vector, pBI101 (see Figure 9) contains unique restriction sites for Hind III, Sal I, Xba I, BamH I, and Sma I upstream of the AUG initiator codon of GUS, to which promoter DNA fragments can be conveniently ligated. The cauliflower mosaic virus 35S promoter (see reference 50) as described in the expression vector pROK1 (see reference 51) was ligated into the Hind III and BamH I sites to create pBI121. Similarly the promoter from a tobacco gene encoding the small subunit of ribulosebisphosphate carboxylase small, Ntss23 (see reference 52) deleted of rbcS coding sequences was fused to pBI101 to make pBI131.

Figure 9 illustrates the structure of the expression vectors.

The lower portion of Figure 9 shows the T-DNA region of pBI101, containing polylinker cloning sites upstream of the beta-glucuronidase gene, followed by the nopaline synthase polyadenylation site (NOS-ter). Pst I and Sph I are not unique to the polylinker. The expression cassette is within pBIN 19, giving pBI101 a total length of approximately 12 kb.

The middle portion of Figure 9 shows chimaeric CaMV 35S-GUS gene in pBI121. An 800 bp Hind III - BamH I CaMV 35S promotor fragment (see reference 59) was ligated into the corresponding sites of pBI101. The mRNA initation site is approximately 20 bp 5' of the GUS initiator codon.

The top portion of Figure 9 shows chimaeric rbcS-GUS gene in pBI101. A 1020 bp Hind III - Sma 1 fragment containing the promoter of a tobacco ribulose bisphosphate caarboxylase small subunit gene (rbcS) was ligated into

the corresponding sites of pBI101. The mRNA initiation site is approximately 55 bp 5' of the GUS initiator codon, and contains nearly the entire untranslated leader of the rbcS gene.

Figure 9 also illustrates the differences between the 3 pBI101 plasmids.

Chimaeric GUS genes are expressed in transformed plants

Nicotiana tabacum var. Samsun plants were transformed with Agrobacterium binary vectors (see reference 45) containing transcriptional fusions of either the CaMV 35S promotor or the tobacco rbcS promotor with the coding region of GUS as shown in Figure 9. Several kanamycin resistant plants were regenerated from each transformation.

Two rbcS-GUS transformants and two CaMV-GUS transformants were chosen for further study. First assays were made of various organs of one plant from each transformation, axenically cultured in 3000 lux white light, 18 hour day, 6 hour night. Extracts were prepared from axenic tobacco plants using about 50 mg fresh weight of tissue ground in 500 ul extraction buffer. 5 ul of extract was assayed as described in "Materials and Methods" above. Mature leaves were lower, expanded leaves approximately 80 mm long, while young leaves were approximately 5 mm long, and were dissected from the shoot apex. All samples were taken from the same plant (either CaMV-GUS 21, rbcS GUS 2 or non-transformed) at the same time. Leaf tissue was taken from a non-transformed plant for this assay, although all organs showed no GUS activity (date not shown).

The results of this analysis are shown in Figure 10, and

tabulated in Table 1 using either of two normalisation methods (see following discussion).

The rate data shown in Figure 10 were converted to specific activity by measuring the protein concentration of the extracts using the Bradford reagent. The data are also presented as GUS activity per unit weight of DNA in the extract to better account for the differences in cell number between different tissues.

The plant containing a rcbS-GUS fusion (rbcS-GUS 2) exhibited a pattern of gene expression consistent with earlier studies using heterologous rbcS gene fusions (see reference 53). The highest specific activity, using either protein or DNA as a denominator, was found in older leaves (about 8cm long), with progressively less activity in very young leaves (less than 5 mm), stems and roots. The other rbcS-GUS fusion plant showed a similar pattern (data not shown).

The two plants transformed with the CaMV 35S-GUS fusion displayed a pattern of gene expression distinct from that of the rbcS-GUS fusion plants. The highest levels of activity were found in roots, with similar levels in stems. GUS activity was also high in leaves, consistent with previous observations that the CaMV 35S promoter is expressed in all plant organs (see reference 50).

To verify that no significant rearrangements of the transforming DNA had occurred, a Southern blot analysis was conducted as shown in Figure 11, which is an autoradiograph of a Southern blot of DNA extracted from transformed plants and digested with restriction endonucleases. The filter was hybridised with a ^{32}p

labelled restriction fragment containing the coding region of the beta-glucuronidase gene. In this Figure

Lane 1. CaMV-GUS 21 EcoR I
Lane 2. CaMV-GUS 21 EcoR I & Hind III
Lane 3. CaMV-GUS 29 EcoR I
Lane 4. CaMV-GUS 29 EcoR I & Hind III
Lane 5. rbcS-GUS 2 EcoR I
Lane 6. rbcS-GUS 2 EcoR I & Hind III
Lane 7. rbcS-GUS 5 EcoR I
Lane 8. rbcs-GUS 5 EcoR I & Hind III
Lane 9. Non-transformed EcoR I
Lane 10. Non-transformed EcoR & Hind III
Lane 11. Single copy reconstruction of GUS coding region
Lane 12. Five copy reconstruction.

Digestion of DNA extracted from all of the transformants with Hind III and EcoRI released a single internal fragment of T-DNA consisting of the nopaline synthase polyadenylation site, the GUS coding region and the promoter (CaMV35S or rbcS). RbcS-GUS transformants contained 3 copies (rbcS-GUS2, Figure 11, lane 6) and about 7 copies (rbcS-GUS5, lane 8) of the predicted 3.1 kb Hind III - EcoR 1 fragment. Digestion with EcoRI revealed multiple border fragments (Figure 11, lanes 5 and 7) confirming the copy number estimates deduced from the double digestions. Similarly CaMV 35S-GUS plants had multiple insertions as shown in Figure 11 lanes 1 to 4. CaMV-GUS 21 had 3 copies of the predicted 2.9 kb fragment, while CaMV-GUS29 had 2 copies. No hybridisation of the labelled GUS coding region to untransformed plant tissue was observed (lanes 9 and 10).

Visualisation of GUS activity on SDS-polyacrylamide gels

Extracts of transformed plants were prepared and electrophoresed, together with negative controls and varying amounts of purified beta-glucuronidase, on an SDS-polyacrylamide gel. The gel was rinsed to reduce the SDS concentration, and then treated with the fluorogenic susbstrate, MUG. After the reaction has progressed sufficiently, the gel was made alkaline to enhance fluorescence, placed on a long wave UV box and photographed (Figure 12). The gel was trans-illuminated with 365 nm light and photographed using a Kodak Wratten 2E filter. In Figure 12,

Lane 1. Transformed plant extract - CAB-GUS fusion
Lane 2. Transformed plant extract - SSU-GUS 2
Lane 3. Transformed plant extract - CaMV-GUS 21
Lane 4. Non-transformed plant extract
Lane 5. Non-transformed plant extract plus 1 ng GUS
Lane 6. Non-transformed plant extract plus 10 ng GUS
Lane 7. Non-transformed plant extract plug 50 ng GUS.

GUS activity can be seen in all lanes containing purified enzyme, with a limit of sensitivity in this experiment of one nanogram. In other experiments, we have observed activity with as little as 0.2 ng has been observed. The lanes containing the SSU-GUS and CaMV-GUS fusion extracts show GUS activity that migrates with the same mobility as the purified enzyme, indicating that translation is initiating and terminating at the correct locations in the GUS sequence and tht no significant post-translational processing is occuring. An additional lane was included that contained a protein fusion between part of the tobacco chlorophyll a/b binding protein and GUS that shows decreased mobility relative to purified beta-glucuronidase, as predicted. Staining of gels using the

histochemical methods described below proved to be effective, but not as sensitive as the fluorogenic stain (data not shown).

GUS activity in plants can be visualised using histochemical methods

Although there are very few organs in plants, each organ is composed of many different cell types, often associated in the form of distinct tissues. Since different organs consist of unequal combinations of these cell types intermingled in a highly complex fashion, the meaningful interpretation of "organ-specific" gene expression becomes a difficult exercise. One approach to characterising cell-type specific expression of chimeric genes in plants has utilised microdissection (see reference 54). These methods are, however, extremely laborious, prone to varying degrees of contamination, and many cell-types within plants are inaccessible to the techniques. Alternatively, localisation of chimeric gene activity by histochemical methods has been successful in other systems (see eg reference 2).

To determine whether it would be possible to use histochemistry to investigate single-cell or tissue-specific expression of GUS gene fusions in plants, preliminary experiments were carried out on sections of stems of several independently transformed rbcS-GUS and CaMV-GUS plants. Stem sections were chosen both for their ease of manipulation and because most of the cell types of a mature plant are represented in stem. To illustrate the light-regulated nature of the rbcS-GUS fusion, the plants were illuminated from one side only for one week before sectioning. Sections from both plants stained intensely

with the substrate while non-transformed tissue did not stain. Stem sections of CaMV-GUS plants always show highest levels of activity in phloem tissues along the inside and outside of the vascular ring, most prominently in a punctate pattern that overlies the internal phloem and in the rays of the phloem parenchyma which join the internal and external phloem (see reference 55). There is also variable lighter staining throughout the parenchymal cells in the cortex and in the pith, and also in epidermal cells, including the trichomes.

RbcS-GUS stem sections rarely if ever show intense staining in the trichomes, epidermis, vascular cells or pith, but tend to stain most intensely over the cortical parenchyma cells containing chloroplasts (chlorenchyma), with faint and variable staining in the pith. Although the strongest staining is most often seen in a symmetrical ring around the vascular tissue just inside the epidermis, an asymmetric distribution of staining in the cortical stem cells is sometimes observed. Suspecting that this pattern was due to uneven lighting, a plant was illuminated from one side for one week before sectioning, and it was found that the staining was asymmetric, with intense staining in the chloroplast-containing cells proximal to the light source. The staining patterns observed for both the CaMV 35S-GUS and the rbcS-GUS transformants are consistent between several independent transformants. Untransformed plants never show staining with X-Gluc, even after extending assays of several days.

DISCUSSION

New methods are provided for analysing gene expression in transformed plants that are potentially of general

utility. The beta-glucuronidase gene from E. coli has been expressed at high levels in transformed tobacco plants with no obvious ill effects on plant growth or reproduction. The ability to quantitate gene expression through the routine use of enzyme kinetics greatly enhances the precision and resolution of the question that can be asked. It should be emphasised that the determination of rates of enzyme activity eliminates the vagaries inherent in CAT, NPTII and luciferase assays, and allows accurate determination of quantity of chimaeric gene production, even over an intrinsically fluorescent background. The fluorometric assay is very specific, extremely sensitive, inexpensive and rapid. Minute quantities of tissue can be assayed with confidence; recently GUS levels have been measured in isolated single cells of transformed plants.

Beta-glucuronidase is very stable in extracts and in cells, with a half-life in living mesophyll protoplasts of about 50 hours. Because of this, it is felt reasonable to interpret GUS levels as indicative of the inegral of transcription and translation, rather than the rate. In addition, GUS is not completely inactivated by SDS-PAGE, can tolerate large amino-terminal fusions without loss of enzyme activity and can be transported across chloroplast membranes with high efficiency. It is felt, therefore, that the system will also be very useful in studying the transport and targeting of proteins, not only in plants, but in other systems that lack intrinsic beta-glucuronidase activity, such as Saccharomyces cerevisiae and Drosophila melanogaster.

A commercially available histochemical substrate has been used to demonstrate GUS activity in transformed plant

tissue. Other substrates are available and give excellent results. It is emphasised that meaningful interpretation of results of histological analysis in terms of extent of chimaeric gene activity, whether by in situ hybridisation methods or by histochemistry, as presented here, is not a trivial or straightforward matter. There are numerous variables that must be dealt with (reviewed in reference 18). However, with these cautions, histochemical methods can be very powerful for resolving differences in gene expression between individual cells and cell-types within tissue.

A distinctly non-uniform distribution of GUS activity in stem sections of several CaMV-GUS transformed plants has been observed. Different cell-types within plants are expected to have differing metabolic activity with corresponding differences in rates of transcription and translation, and our results may reflect such a difference. Alternatively, since many of the cells of the phloem have very small cross-sectional areas, the intense dye deposition seen in these regions may simply reflect the greater cell number per unit area. The localisation that is observed may also be due to a real difference in the level of expression of the CaMV 35S promoter between cell types. Recently, Nagata et al (see reference 62) have argued that the CaMV 35S promoter is preferentially active in cells during the S phase of the cell cycle. If this is true, then the pattern of GUS staining observed may reflect cell division activity in these cells. This observation is consistent with the proposed role of the 35S transcript of CaMV in viral replication (reference 63). It is also interesting that the other class of plant DNA viruses, the geminiviruses, replicates in the phloem parenchyma (reference 64). It is concluded therefore that

it is no longer adequate to describe the 35S promoter as "constitutive" solely by the criteria of expression in all plant organs, when there may be a strong dependence of transcription on cell-type or cell cycle.

The distribution of GUS activity in the stem sections of plants transformed with rbcS-GUS genes is consistent with data that indicate a requirement for mature chloroplasts for maximal transcription of chimaeric rbcS genes (see eg reference 54). Cortical parenchymal cells in the stem contain varying numbers of chloroplasts, while those in the pith and epidermis of the stem rarely contain chloroplasts.

Different cell-types present in each organ contribute differently to the patterns of gene expression, and each organ consists of different proportions of these cell-types. It has been undertaken to minimise this effect on quantitative analysis of extracts by suitable choice of a denominator. The parameter that needs to be studied with gene fusions is most often the expression of the gene fusion in each cell. When preparing homogenates from plant organs, the number of cells that contribute to the extract will vary, as will the protein content of each cell and cell-type. The DNA content of the extract will reflect the number of cells that were lysed (see reference 58) whereas the traditional denominator, protein concentration, will not. For example, a single leaf mesophyll cell contains much more protein than a single epidermal cell or root cortical cell. However, each will have the same nucleus with the same potential to express the integrated gene fusion.

Using this approach, it is found that the differential

expression of the rbcS-GUS fusion is much more pronounced between immature and mature leaf when GUS activity is expressed, per mg of DNA (see Table 1). When protein concentration is used as a denominator, the massive induction of GUS activity during leaf maturation is masked by the concomitant induction of proteins involved in photosynthesis.

The observation that the specific activity of GUS produced by CaMV-GUS fusions is the same in immature and mature leaves when expressed using a protein denominator indicates that the rate of GUS accumulation closely follows the rate of net protein accumulation. The two-fold difference in GUS specific activity using a DNA denominator illustrates the accumulation of GUS per cell over time. This quantitative analysis, together with histochemical data, may indicate that the differences between GUS activity in the leaf, stem and root of CaMV-GUS fusion plants could reflect the larger proportion of phleom-associated cells in roots and stems compared to leaves. It is felt that the choice of a DNA denominator best reflects the expression per cell, and hence is a more accurate reflection of the true regulation of the gene.

Prospects of further development of the GUS system

There are many important questions arising from the use of currently available gene-transfer techniques in plants that can be addressed with this new technology. Both Agrobacterium-mediated transformation and direct DNA uptake methods result in cells and plants transformed with varying numbers of integrated copies of the foreign DNA and with different sites of integration, resulting in plants expressing different amounts of chimaeric gene

product (see eg references 65 and 66). Previously, analysis of gene expression in transformed plants has been sufficiently laborious to preclude quantitative assays of the large numbers of plants necessary to finaly delineate the contributions of local integration site and copy number to the expression of transformed genes. Using the methods described here, it will be feasible to quantitate the variation that is often ascribed to differing sites and copy numbers of integrations, and obtain statistically significant answers to these questions.

The availability of routine histochemical analysis will greatly facilitate studies of the mechanism of transformation both by Agrobacterium and by direct DNA methods, as well as permitting a more detailed study of developmental regulation. These methods will also allow very rapid and sensitive screening of transformed cells and tissues. Using the indigogenic substrate X-Gluc, GUS activity from single cells and small cell clusters from suspension cultures can be easily resolved.

GUS assay systems lend themselves very well to automation. The existing spectrophotometric and fluorogenic assays, and new assays using fluorogenic substrates that fluoresce maximally at neutral pH will allow the use of automatic microtitre plate analysis of very large numbers of samples. The activity of GUS in lysed single cells can be measured with accuracy; using new fluorogenic substrates, an analysis of GUS expression in single cells of transformed plants using the fluorescence activated cell sorter is being conducted.

The GUS fusion system has also been used successfully to monitor the transient expression of chimaeric genes

introduced into plant cells via electroporation and/or polyethylene glycol treatment. The sensitivity is found to be very high, allowing expression to be reliably measured from a very small number of cells.

Because of the lack of intrinsic beta-glucuronidase activity in all plants thus far assayed, and because the synthesis of beta-glucuronides can be relatively straightforward, the use of the GUS system to begin "fusion genetics" is being pursued. Due to the complex genomes and long generation times of higher plants, fine scale genetic analysis of complex processes is unfeasible by convention means. However, by using the GUS system and novel substrates, it may be possible to generate positive and negative selections for GUS activity, thereby selecting mutations in the activity of gene fusions, both in planta and in tissue culture.

Table 1

GUS Specific Activity

Gene Fusion: Plant organ	(pmoles 4-MU/min/mg protein) CaMV 35S-GUS	rbcS-GUS	untransformed	(pmoles 4-MU/min/mg DNA) CaMV 35S-GUS	rbcS-GUS
Leaf (5 mm)	283	205	< 0.1	2,530	4,400
Leaf (70 mm)	321	1,523	< 0.1	5,690	93,950
Stem	427	260	< 0.1	13,510	2,650
Root	577	62	< 0.1	12,590	690

References

1. Cox, G.N., and Hirsh, D. (1985) Mol. Cell. Biol. 5, 363-372.

2. Lis, J.T., Simon. J.A., and Sutton, C.A. (1984) Cell 35, 403-410.

3. Beckwith, R.R. and Zipser, D. eds. (1970) The Lactose Operon (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

4. Depicker, A., Stachel, S., Dhaese, P., Zambryski, P. and Goodman H. (1982) J. Molec. Applied Genetics 1, 561-575

5. Bevan, M., Barnes, W. and Chilton, M-D (1983) Nucl. Acids. Res. 11, 369-385.

6. DeGreve, H., Dhaese, P., Seurinck, J., Lemmers, M., Van Montagu, M., and Schell J., (1982) J. Mol. Applied Genetics. 1, 499-513.

7. Otten, L.A.M.B. and Schilperoot, R.S. (1978) Biochem. Biopys. Res. Commun. 527, 497-500.

8. Jones, J.D.G., Dunsmuir, P. and Bedbrook, J. (1985) EMBO J. 4, 2411-2418.

9. Shaw, C.H., Sanders, D.M., Bates, M.R. and Shaw C.H. (1986) Nucl. Acids. Res. 14, 6603-6613.

10. Herrera-Estrella, L., Depicker.A., VanMontagu, M. and

Schell, J. (1983) Nature 303, 209-213.

11. Herrera-Estrella, L., De Block, M.N., Messens, E., Hernalsteens, J.P., van Montagu, M. and Schell J.H (1983) EMBO J. 2, 987-995.

12. Bevan. M.W., Flavell, R.B. and Chilton M-D. (1983) Nature 304, 184-187.

13. Fraley, R.T., Rogers, S.G., Horsch, R.B., Saunders, P.S., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L. and Woo, S.C (1983) Proc. Natl, Acad, Sci USA. 80, 4803-4807.

14. Van den Broeck, G., Timko, M., Kausch, A.P., Cashmore, A.R., van Montagu, M. and herrera-Estrella, L. (1985) Nature 313, 358-363.

15. Gorman, C.M., Moffatt, L.F., and Howard, B.H., (1982) Mol, Cell. Biol. 2, 1044.

16. Reiss, B., Sprengel, R., Will, H., and Schaller, H., (1984) Gene 30, 217-223.

17. Paigen, K. (1979) Ann. Rev. Genet. 13, 417-466.

18. Pearse, A.G.E. (1972) Histochemistry: Theoretical and Applied Third Edition, Vol II, pp 808-840 (Churchill Livingstone, Edinburgh).

19. Novel, G., and Novel, M. (1973) Mol. Gen. Genet. 120, 319-335.

20. Novel, G., Didier-Fichet, M.L., and Stoeber, F. (1974)J. Baceriol. 120, 89-95.

21. Novel, M., and Novel, G. (1976) J. Bacteriol. 127, 406-417.

22. Novel, M., and Novel, G. (1976) J. Bacteriol. 127, 418-432.

25. Birnboim, H.C. and Doly, J. (1979) Mucleic Acids Res. 7, 1513.

26. Maniatis, T., Fritsch, E.F. and Sambrook, J., eds. (1982) Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

27. Dretzen, G., Bellare, M., Sasson-Corsi, P., and Chambon, P. (1981) Anal. Biochem. 112, 295-298.

28. Sanger, F. and Coulson, A.R. (1975) J. Mol. Biol. 95, 441.

29. Biggin, M.D., Gibson, R.J., and Hong, G.F. (1983) Proc. Natl, Acad, Sci, USA 80, 3963-3965.

30. Zoller, M.J., and Smith, M. (1982) Nucleic Acids Res. 10, 6487-6500.

31. Vieira, J. and Messing, J. (1982) Gene 19, 259-268.

32. Dente, L., Cesarini, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645.

33. Bradford, M. (1976) Anal. Biochem. 72, 248.

34. Laemmli, U.K. (1970) Nature 227, 680.

35. Kozak, M. (1983) Microbiol. Rev. 47, 1-45.

36. Stoeber, F. (1961) These de Docteur es Sciences, Paris

37. Kramer, J.M., Cox, G.N., & Hirsh, D., (1982) Cell, 30 599-606.

38. Kramer, J.M., Cox, G.N., & Hirsh, D., (1985) J. Biol. Chem, 260, 1945-1951.

39. Klass, M., Kindsley, S., and Lopez, L., (1984) Mol. Cell. Biol. 4, 529-537.

40. Klass, M.R., and Hirsh, D., (1982) Develom Biol. 84, 299-312.

41. Stinchcomb, D., Shaw, J., Carr, S.H., and Hirsh, D., (1985), Mol. Cell. Biol, 5, 3484-3496.

42. Sebastiano, M., D'Allessio, M., and Razzicalupo, P., (1986), Genetics, 112, 459-468.

43. Fishman, W.H., and Goldman, S.S., (1965), J. Histochem, Cytochem, 13, 441-447.

44. Fire, A., (1986), EMBO J. In the press.

45. Bevan, M.W., (1984) Nucl, Acids Res. 12, 8711-8721.

46. Horch, R.B., Fraley, R.T., Rogers, S.G., Sanders,

P.R., Lloyd, A., and Hoffmann, N., (1984) Science, 223, 496-498.

47. Marashige, T., and Skoog, F., (1962), Physiol. Plant, 15, 473.

48. Guilbault, G., (1973) "Practical Fluorescence: Theory, Methods and Techniques" Dekker, New York.

49. O'Brien, T.P., and McCully, M.E., (1981) "The Study of Plant Structure: Principles and Selected Methods" Termarcarphi Pty. Ltd, Melbourne, Australia.

50. O'Dell, J.T., Nagy, F., and Chua, N-H. (1985) Nature 313, 810-812.

51. Baulcombe, D.C., Saunders, G.R., Bevan, M.W., Mayo, M.A., and Harrison, B.D., (1986) Nature 321, 446-449.

52. Mazur, B.J., and Chui, C-F. (1985) Nucl. Acids Res. 13, 2373-2386.

53. Simpson, J., Van Montagu, M., and Herrera-Estrella, L., (1986) Science 233, 34-38.

54. Simpson, J., Schell, J., Van Montagu, M., and Herrera-Estrella, L., (1986) Nature 323, 551-554.

55. Esau, K., (1977) The Anatomy of Seed Plants, Wiley, New York.

56. Mones, D.S. and Quigley, M. (1981) Anal. Biochem. 114, 193-201.

57. Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266-267.

58. Labarca, C. and Paigen, K. (1980) Anal. Biochem. 102, 344-352.

59. Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards K.E. (1982) Cell 30, 763-773.

60. Ow, D.W., Wood, K.V., DeLuca, M., DeWet, J.R., Helsinki, D.R. and Howell, S.H. (1986) Science 234, 856-859.

61. DeLuca, M., and McElroy, W.D. (1978) Methods in Enzymology 57, 3-15.

62. Nagata, T., Okada, K., Kawazu, T. and Takebe, I. (1987) Mol. Gen. Genet. 207, 242-244.

63. Pfeiffer, P. and Hohn, T. (1983) Cell 33, 781-790.

64. Kim, K.S., Shock, T.C. and Goodman, R.M. (1978) Virology 89, 22-23.

65. Jorgensen, R.A. Snyder, C. and Jones, J.D.G. (1987) Mol. Gen. Genet. 207, 471-477.

66. Jones J.D.G., Gilbert, D.E., Grady, K.L. and Jorgensen R.A. (1987) Mol. Gen. Genet. 207, 478-488.

Claims

1. A gene fusion product comprising a gene coding for beta-glucuronidase (GUS).

2. A gene fusion product according to claim 1, comprising the uidA gene of E. coli.

3. A gene fusion product according to claim 1 or 2, wherein the gene coding for GUS is under control of the E. coli lacZ promoter.

4. A gene fusion product according to claim 1 or 2, comprising the cauliflower mosaic virus (CaMV) 35S promoter.

5. A gene fusion product according to claim 1 or 2, comprising the promoter from the small subunit of ribulose bisphosphate carboxylase (rbcS).

6. A gene fusion product according to any one of the preceding claims, further comprising a gene of interest.

7. A plasmid comprising a gene coding for GUS.

8. A plasmid according to claim 7, constructed from plasmid pBIN19.

9. A plasmid according to claim 7 or 8, comprising pBI101.

10. A plasmid according to claim 7, comprising pBI121.

11. A plasmid according to claim 7, comprising pBI131

12. A plasmid according to any one of claims 7 to 11, further comprising a gene of interest.

13. A method of introducing a gene of interest to a host, comprising introducing to the host a gene fusion product comprising a gene coding for GUS and the gene of interest.

14. A method of monitoring expression of a gene of interest in a host, comprising introducing to a host a gene fusion product comprising a gene coding for GUS and the gene of interest, and monitoring to detect the presence of GUS.

15. A method according to claim 14, wherein GUS is assayed using spectrophotometric, fluorometric or histochemical techniques.

16. A method according to claim 13, 14, or 15 wherein the host comprises a plant, animal, yeast or other micro-organism.

17. A transformed host to which has been introduced a gene fusion product comprising a gene coding for GUS.

18. A product of the transformed host of claim 17.